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(54) Title: USE OF PLATELET ACTIVATING FACTOR (PAF) INHIBITORS TO INHIBIT IL-5 INDUCED EOSINOPHIL ACTIVATION OR DEGRANULATION

(57) Abstract

A therapeutic method is provided to inhibit or treat pathologies associated with eosinophil activation, adherence and/or degranulation. The method comprises the administration to a mammal in need of said therapy an effective amount of an inhibitor of platelet activating factor (PAF).

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USE OF PLATELET ACTIVATING FACTOR (PAF) INHIBITORS TO INHIBIT IL-5 INDUCED EOSINOPHIL ACTIVATION OR DEGRANULATION

Statement of Government Rights

This invention was made with a grant from the Government of the United States of America (Grant Nos. AI34486 and AI34577 from the National Institutes of Health). The Government may have certain rights in the invention.

Background of the Invention

Eosinophils play important roles in the pathophysiology of asthma and other inflammatory diseases. Activated eosinophils adhere to endothelial cells and subsequently release inflammatory mediators (Lee et al., 1984; Gleich et al., 1994), superoxide anion (Sedgwick et al., 1988) and cationic granule proteins (Abu-Ghazaleh et al., 1989). The toxic granule proteins include major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). These cationic proteins play a role in the pathophysiology of bronchial asthma (Evans et al., 1997a; Lefort et al., 1996). In addition to superoxide anion, the eosinophil can generate reactive oxygen intermediates, including hydrogen peroxide, hydroxyl radical and singlet oxygen, large quantities of leukotrienes C_4 and D_4, platelet-activating factor (PAF), as well as a series of arachidonic intermediates including 5-hydroxyeicosatetraenoic acid (5-HETE), 5, 15 and 8,15-diHETE, prostaglandin E2 and thromboxane B2. The eosinophil also possesses a series of enzymes, collagenase, Charcot-Leyden crystal protein, and the 92 kDa gelatinase (MMP-9), which appear to play a role in eosinophil migration through tissues.

And in recent years, considerable attention has been paid to the ability of eosinophils produce cytokines, and evidence has come forth in the literature that interleukin (IL)-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-16, RANTES, MIP-1α, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), transforming growth factor-α (TGF-α), TGF-β and platelet-derived growth factor (PDGF) are produced by eosinophils. At the molecular level, eosinophil proliferation and differentiation are regulated by various cytokines, such as IL-3, IL-5 and GM-CSF. See Silberstein et al., 1989.

In particular, IL-5 is known to play an important role in the regulation of the immune system and is one of several cytokines regulating organ-specific eosinophil infiltration and degranulation during normal host immune function (Kita et al., 1992). Moreover, IL-5, as well as the cytokines IL-3, IFN-γ and GM-CSF, prolong the survival of eosinophils *in vitro* (Valenus et al., 1990) and augment eosinophil function (Rothenberg et al., 1988; Fujisana et al., 1990); Silberstein et al., 1986). Furthermore, several types of diseases are correlated with IL-5 activity, including parasitic, autoimmune, atopic and malignant diseases.

IL-5 also primes eosinophils for enhanced locomotor responses to chemotactic agents, such as PAF, leukotriene B4, and IL-8 (Sehmi et al., 1992). Recent information indicates that IL-5 is present in the lung following allergen-induced pulmonary late allergic reactions (Sedgwick et al., 1991), and mRNA for IL-5 is expressed in the bronchial epithelium of patients with asthma (Hamid et al., 1991). These observations suggest that the inflammation associated with asthma is dependent on the presence of cytokines, especially IL-5, and recent data showing that antibodies to IL-5 block both antigen-induced eosinophilia and antigen-induced bronchial hyperactivity support that view (Mauser et al., 1992).

Although eosinophils produce a number of inflammatory mediators, eosinophil-associated inflammation and tissue injury are likely due to toxic effects of the granule proteins (Gleich et al., 1994). Eosinophil infiltration and deposition of granule proteins have been observed in bronchoalveolar lavage (BAL) fluids and in bronchial tissues obtained from patients with bronchial asthma (Broide et al., 1991; Filley et al., 1982). Both *in vitro* and *in vivo* studies have supported the conclusion that eosinophil granule proteins damage respiratory epithelium (Motojima et al., 1989; Frigas et al., 1986). Eosinophil degranulation can be stimulated with Ig (Abu-Ghazaleh et al., 1989), lipid mediators (Kroegel et al., 1989) and cytokines (Horie et al., 1994). Furthermore, eosinophils stimulated with cytokines (Triggiani et al., 1992), calcium ionophore (White et al., 1993), fMLP (White et al., 1993) and IgG-coated sepharose beads (Cromwell et al., 1990), have been shown to release PAF.

As a biologically active phospholipid (Prescott et al., 1990), PAF activates platelets, eosinophils and neutrophils, and has been implicated in the pathophysiology of asthma, inducing bronchoconstriction, airway microvascular leakage, edema, and bronchial hyperresponsiveness (Chung et al., 1991). PAF stimulates eosinophil adhesion (Kimani et al., 1988; Lamas et al., 1988) and causes eosinophils to release superoxide anion and eosinophil granule proteins such as eosinophil-derived neurotoxin (EDN) (Kroegel et al., 1988). It is also a potent chemotactic agent (Wardlaw et al., 1986) and causes an increase in cytosolic free calcium in eosinophils (Kernen et al., 1991). PAF also may act as a secondary messenger for activation of leukocytes (Stewart et al., 1990).

Eosinophil adhesion and PLA_2 activation are prerequisites for activated eosinophil degranulation. Recently, β_2-integrin-dependent adhesion was shown to be necessary for degranulation of eosinophils activated with GM-CSF, IgG, PAF and PMA (Horie et al., 1997; Kaneko et al., 1995; Horie et al., 1994). PLA_2 is an enzyme that initiates the synthesis of PAF via the remodeling pathway by catalyzing the hydrolysis of a phospholipid precursor (Prescott et al., 1990). Mepacrine inhibits this pathway (Vigo, 1980; Jain et al., 1984). Because mepacrine inhibits the release of EPO, LTC_4, and superoxide from eosinophils stimulated with fMLP plus cytochalasin b, PLA_2 likely plays a role in these functions (White et al., 1993).

Glucocorticoids are the most useful class of drugs for treating many eosinophil-related disorders, including bronchial asthma (Schleimer et al., 1990). Glucocorticoids, e.g., dexamethasone, methylprednisolone and hydrocortisone, produce eosinopenia in normal persons, decrease circulating eosinophils in patients with eosinophilia, and reduce eosinophil influx at inflammatory sites (Butterfield et al., 1989). The mechanism of these effects is still uncertain.

For many patients with asthma, glucocorticoids are the principal therapy and these patients may require glucocorticoid therapy for long periods of time, e.g., months to years. In fact, the disease can be characterized as one of chronic glucocorticoid toxicity, in that the toxicity of these steroids can cause severe morbidity and even mortality in the patients. Furthermore, cessation of glucocorticoid therapy leads to withdrawal symptoms, such as malaise and

muscle pain. However, presently, glucocorticoids are the only effective therapy for severe asthma, and are prescribed long-term despite their toxicity.

Thus, a need exists for improved therapeutic methods to treat eosinophil-associated indications, diseases or pathologies, such as bronchial asthma, with agents that inhibit or reduce the degranulation of eosinophils.

Summary of the Invention

The present invention provides a therapeutic method which counteracts or prevents eosinophil-associated pathologies mediated by cytokines, such as IL-5, growth factors, such as GM-CSF, or by binding of immunoglobulins. The method comprises the administration of one or more agents that are inhibitors of platelet activating factor (PAF), e.g., a PAF receptor antagonist, to an afflicted mammal, e.g., a human, in an amount effective to inhibit or reduce eosinophil activation and/or degranulation. Thus, the agents of the invention inhibit or reduce (antagonize) the activity of cytokines, growth factors and/or immunoglobulins on eosinophils which, in turn, limits or blocks the pathogenic effects of the proteins secreted and/or released by eosinophils on the tissue of the mammal in need of said treatment. Inhibitors of PAF include, but are not limited to, agents that reduce or block the synthesis of PAF, the secretion of PAF, the binding of PAF to a receptor on eosinophils, or the intracellular signal generated by the binding of PAF to a receptor on eosinophils, although the invention is not limited to an agent which has a particular mechanism of action. Preferred agents are those which are permeant to eosinophils, e.g., they are intracellular inhibitors of the PAF synthetic or signaling pathway.

As described hereinbelow, autocrine PAF is an essential mediator in eosinophil effector functions, e.g., degranulation and superoxide production, induced by either IL-5 or immobilized Ig, as shown by the effect of PAF, an agonist of eosinophils, released by activated eosinophils on the magnitude of the same eosinophils' response to the original stimuli. Thus, human eosinophils produced superoxide and released granule proteins when stimulated with immobilized IgG or soluble IL-5. Moreover, substantial amounts of PAF were detected in the supernatants of eosinophils stimulated with IgG or IL-5. These eosinophil responses were inhibited by CV6209, a competitive PAF receptor

antagonist, and pertussis toxin (PTX), suggesting important roles of PAF and PTX-sensitive G-proteins for eosinophil responses to IgG or IL-5. Two other PAF antagonists, structurally unrelated to each other, also inhibited IL-5-mediated eosinophil function. Surprisingly, two other PAF antagonists, WEB 2086 and SM-10661, did not inhibit IL-5-mediated eosinophil function. Furthermore, PAF production by activated eosinophils was dependent on β_2-integrins, although it is also envisioned that the agents of the invention can inhibit adhesion-independent degranulation. Together, these findings suggest that eosinophils likely use a PAF-mediated autocrine system to amplify the signals generated by Fcγ receptors or cytokine receptors.

The invention also provides a method to inhibit or treat an eosinophil-associated pathology, e.g., bronchial asthma. The method comprises administering to a mammal in need of said therapy an amount of an inhibitor of platelet activating factor effective to counteract at least one symptom of the pathology. Preferably, the inhibitor is an inhibitor of the platelet activating factor receptor. To treat bronchial asthma, it is preferred that the inhibitor is administered to the respiratory tract of the mammal. Also, such a method may be useful for studying the mechanism of action of other therapeutic agents or potential therapeutic agents that effect eosinophil effector function(s) so as to further elucidate the beneficial or pathological effects of eosinophils in biological systems.

Also, as described hereinbelow, it was observed that IL-5 induced degranulation of eosinophils was inhibited by a PAF receptor antagonist in the absence of adherence, i.e., degranulation was not mediated by PAF. This was unexpected, as eosinophil degranulation was believed to be dependent on eosinophil adherence and subsequent PAF secretion.

PAF receptor antagonists include, but are not limited to, CV6209, WEB 2086, kadsurenome, kadsurin B, UK74505, SM-10661, BN52063, SR27417A, CV3988, ONO-6240, ginkolide B, etizolam, Y24180, Cl66985, U66985, methyl-WEB 2086, Y24180, BN52111, desmethyl-Y24180, SRI63-072, R019-3704, SDZ64-412, L-652,731, 48740RD, BN52021 and analogs of PAF such as those described in Grigoriadis et al. (1991) and Tokumura et al. (1985). For the treatment of bronchial asthma, it is preferred that PAF

antagonists such as Y24180 and SR24717A are not orally administered. In certain embodiments of the invention, it is preferred that the PAF receptor antagonist is not CV6209.

Other inhibitors of PAF include, but are not limited to, inhibitors of PLA_2 such as mepacrine, local anesthetics, phenothiazines, peptides related to lipocortin, 7,7-dimethyleicosadienoic acid, quinacrine and aristolochic acid.

Brief Description of the Figures

Figure 1 depicts a graph of superoxide production by eosinophils stimulated with PMA, immobilized IgG or IL-5 as a function of mepacrine concentration.

Figure 2 depicts a graph of the amount of PAF released per 10^6 eosinophils, which had been stimulated with IL-5, immobilized IgG or PMA, at 15 minutes and 45 minutes post-stimulation.

Figure 3 shows a graph of the amount of superoxide released by eosinophils stimulated with various amounts of PAF in the presence of increasing amounts of CV6209.

Figure 4 shows the amount of superoxide released by eosinophils over time after stimulation with immobilized IgG (A); IL-5 (B); PMA (C); or PAF (D), in the presence of varying amounts of CV6209.

Figure 5 depicts the superoxide release by eosinophils after stimulation with immobilized IgG (A); IL-5 (B); PMA (C); or PAF (D), as a function of CV6209 concentration.

Figure 6 depicts the release of EDN by eosinophils stimulated with immobilized IgG or IL-5 in the presence or absence of CV6209.

Figure 7 shows the amount of superoxide released by eosinophils stimulated with various agents in the presence or absence of pertussis toxin (PTX).

Figure 8 shows a comparison of the effect of hexanolamine PAF versus Y24180 on superoxide production by eosinophils.

Figure 9 shows a graph of LTC4 release by eosinophils stimulated with IL-5, immobilized IgG or PAF in the presence of CV6209.

Detailed Description of the Invention

Indications Amenable to Treatment by the Agents of the Invention

Conditions, indications or diseases amenable to treatment by the therapeutic agents of the invention include upper and lower respiratory tract diseases, eosinophilic myocitis, Chrohn's disease, renal or hepatic graft rejection, eosinophilic endomyocardial disease, acute necretizing myocarditis, primary biliary cirrhosis, sclerosing cholangitis, retroperitoneal fibrosis, medistinum fibrosis, Ridell's invasive fibrosis, thyroiditis, cirrhosis, urticaria, skin diseases, nasal polyps, and inflammatory diseases, e.g., allergic disorders, that are associated with eosinophilia. Eosinophilia is the infiltration of eosinophils into tissues such as blood or lung, and the activation of those eosinophils, which results in the production of eosinophil-derived proteins that in turn mediate pathogenic effects. The infiltration of eosinophils into tissues, and the subsequent degranulation of the eosinophils, is associated with hypersensitivity diseases such as bronchial asthma, chronic eosinophilic pneumonia, vernal conjunctivitis, allergic conjunctivitis, giant papillary conjunctivitis, allergic rhinitis, allergic sinusitis, and allergic gastroenteropathy. Examples of other eosinophil-associated diseases include eosinophilic gastroenteritis, atopic dermatitis, bullous pemphigoid, episodic angioedema associated with eosinophilia, ulcerative colitis, and inflammatory bowel disease.

Other lung diseases associated with pulmonary eosinophilia include helminth infection, e.g., tropical pulmonary eosinophilia syndrome (TPE), pulmonary infiltrates with eosinophilia (PIE), and pulmonary strongyloidiasis, Churg-Strauss syndrome, allergic bronchopulmonary aspergillosis, chronic obstructive pulmonary disease (COPP), bronchocentric granulomatosis, drug-induced lung disease, acute eosinophilic pneumonia, pulmonary fibrosis, Spanish toxic oil syndrome (TOS), eosinophilia-myalgia syndrome (EMS), eosinophilic granuloma and the hypereosinophilic syndrome (HES). (For a review of lung diseases associated with eosinophilia, see Allen and Davis (1994)).

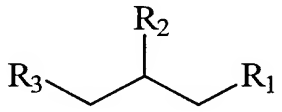
Other diseases characterized by the presence of pulmonary eosinophilic infiltrations include Wegener's granulomatosis, lymphoidmatoid granulomatosis, eosinophilic granuloma of the lung, adult respiratory distress

syndrome, and post-trauma pleural effusions which contain eosinophils or eosinophil containing pleural effusions associated with infections, such as tuberculosis (see Spry, In: Eosinophils, Oxford University Press, pp. 205-212 (1988), the disclosure of which is specifically incorporated by reference herein).

In addition to the diseases discussed above, many other conditions associated with elevated levels of eosinophil activation and accumulation, some of which are presently treated with glucocorticoids, are amenable to treatment by the agents of the invention.

Preferred Therapeutic Agents for Use in the Methods of the Invention

Preferred therapeutic agents include agents that inhibit or block eosinophil activation and/or degranulation that is mediated by a cytokine, a growth factor or immunoglobulins. A preferred agent is a compound of formula I:



I

wherein

R_1 is (C_{14}-C_{22})alkoxy, (C_{14}-C_{22})alkanoyloxy, N-[(C_{14}-C_{22})alkyl]aminocarbonyloxy, (C_{14}-C_{22})alkoxycarbonyloxy, (C_{14}-C_{22})alkylthio, (C_{14}-C_{22})alkanoylthio, N-[(C_{14}-C_{22})alkyl]aminocarbonylthio, or (C_{14}-C_{22})alkoxycarbonylthio;

R_2 is (C_1-C_6)alkoxy, (C_1-C_6)alkylthio, (C_1-C_6)alkanoyloxy, or (C_1-C_6)alkanoylthio;

R_3 is -OP(=O)(OR_a)OR_b, -O-C(=O)R_c, or -O-C(=O)NR_dR_e;

R_a is hydrogen or (C_1-C_6)alkyl;

R_b, R_c, and R_d are each independently -(C_1-C_8)alkyl-R_f or -(C_2-C_8)alkyl-N(R_g)(R_h);

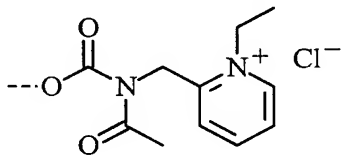
R_e is hydrogen, (C_1-C_6)alkyl, or (C_1-C_6)alkanoyl;

R_f is 2-pyridyl, 3-pyridyl, or 4-pyridyl;

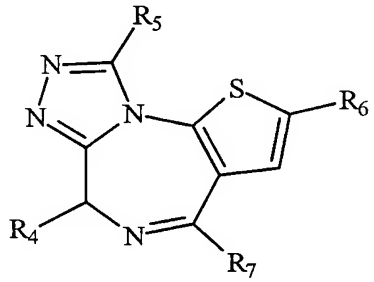
R_g and R_h, are each independently $(C_1$-$C_3)$alkyl; or R_g and R_h together with the nitrogen to which they are attached are pyrrolidino, piperidino, morpholino, imidazolidin-1-yl, or piperazin-1-yl;

or a pharmaceutically acceptable salt thereof.

In certain embodiments of the invention, it is preferred that R_1 is not octadecylaminocarbonyloxy, when R_2 is methoxy and R_3 is:



Another preferred agent for use in the methods of the invention is a compound of formula (II):



II

wherein

R_4 and R_5 are each independently hydrogen, $(C_1$-$C_6)$alkyl, $(C_1$-$C_6)$alkoxy, $(C_1$-$C_6)$alkanoyl, $(C_1$-$C_6)$alkanoyloxy, aryl, or aryl$(C_1$-$C_6)$alkyl; and

R_6 and R_7 are each independently aryl or aryl$(C_1$-$C_6)$alkyl;

wherein any aryl or aryl$(C_1$-$C_6)$alkyl may optionally be substituted by 1, 2, or 3 halo, hydroxy, $(C_1$-$C_6)$alkyl, $(C_1$-$C_6)$alkoxy, $(C_1$-$C_6)$alkanoyl, $(C_1$-$C_6)$alkanoyloxy, trifluoromethyl, trifluoromethoxy, nitro, cyano, or amino;

or a pharmaceutically acceptable salt thereof.

Preferred agents for use in the practice of the methods of the invention include CV6209, Y24180, hexanolamine PAF, and analogs thereof.

The following definitions are used, unless otherwise described: halo is fluoro, chloro, bromo, or iodo. Alkyl, alkoxy, etc. denote both straight and branched groups; but reference to an individual radical such as "propyl" embraces only the straight chain radical, a branched chain isomer such as "isopropyl" being specifically referred to. Aryl denotes a phenyl radical or an ortho-fused bicyclic carbocyclic radical having about nine to ten ring atoms in which at least one ring is aromatic.

It will be appreciated by those skilled in the art that compounds of the invention having a chiral center may exist in and be isolated in optically active and racemic forms. Some compounds may exhibit polymorphism. It is to be understood that the present invention encompasses any racemic, optically-active, polymorphic, or stereoisomeric form, or mixtures thereof, of a compound of the invention, which possess the useful properties described herein, it being well known in the art how to prepare optically active forms (for example, by resolution of the racemic form by recrystallization techniques, by synthesis from optically-active starting materials, by chiral synthesis, or by chromatographic separation using a chiral stationary phase).

Specific values listed below for radicals, substituents, and ranges, are for illustration only; they do not exclude other defined values or other values within defined ranges for the radicals and substituents

Specifically, (C_1-C_6)alkyl can be methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, sec-butyl, pentyl, 3-pentyl, or hexyl; (C_{14}-C_{22})alkyl can be tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, eicosanyl, heneicosanyl, or docosanyl; (C_1-C_6)alkoxy can be methoxy, ethoxy, propoxy, isopropoxy, butoxy, iso-butoxy, sec-butoxy, pentoxy, 3-pentoxy, or hexyloxy; (C_1-C_6)alkylthio can be methylthio, ethoxy, propylthioy, isopropylthioy, butylthio, iso-butylthio, sec-butylthio, pentylthio, 3-pentylthio, or hexylthio; (C_{14}-C_{22})alkoxy can be tetradecyloxy, pentadecyloxy, hexadecyloxy, heptadecyloxy, octadecyloxy, nonadecyloxy, eicosanyloxy, heneicosanyloxy, or docosanyloxy; (C_{14}-C_{22})alkanoyloxy can be tetradecanoyloxy, pentadecanoyloxy,

hexadecanoyloxy, heptadecanoyloxy, octadecanoyloxy, nonadecanoyloxy, eicosanoyloxy, heneicosanoyloxy, or docosanoyloxy; (C_{14}-C_{22})alkoxycarbonyl can be tetradecyloxycarbonyl, pentadecyloxycarbonyl, hexadecyloxycarbonyl, heptadecyloxycarbonyl, octadecyloxycarbonyl, nonadecyloxycarbonyl, eicosanyloxycarbonyl, heneicosanyloxycarbonyl, or docosanyloxycarbonyl; (C_{14}-C_{22})alkylthio can be tetradecylthio, pentadecylthio, hexadecylthio, heptadecylthio, octadecylthio, nonadecylthio, eicosanylthio, heneicosanylthio, or docosanylthio; (C_{14}-C_{22})alkanoylthio can be tetradecanoylthio, pentadecanoylthio, hexadecanoylthio, heptadecanoylthio, octadecanoylthio, nonadecanoylthio, eicosanoylthio, heneicosanoylthio, or docosanoylthio; (C_1-C_6)alkanoyl can be acetyl, propanoyl or butanoyl; (C_1-C_6)alkoxycarbonyl can be methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl, isopropoxycarbonyl, butoxycarbonyl, pentoxycarbonyl, or hexyloxycarbonyl; (C_1-C_6)alkanoyloxy can be formyloxy, acetoxy, propanoyloxy, butanoyloxy, isobutanoyloxy, pentanoyloxy, or hexanoyloxy; (C_1-C_6)alkanoylthio can be formylthio, acetylthio, propanoylthio, butanoylthio, isobutanoylthio, pentanoylthio, or hexanoylthio; and aryl can be phenyl, indenyl, or naphthyl;

A specific value R_1 is (C_{14}-C_{22})alkoxy or N-[(C_{14}-C_{22})alkyl]aminocarbonyloxy. Another specific value for R_1 is N-[(C_{16}-C_{20})alkyl]aminocarbonyloxy. A preferred value for R_1 is (C_{14}-C_{20})alkoxy. Another preferred value for R_1 is N-[(C_{16}-C_{20})alkyl]aminocarbonyloxy.

A specific value for R_2 is (C_1-C_6)alkoxy, or (C_1-C_6)alkanoyloxy.

A specific value for R_3 -OP(=O)(OR_a)OR_b, wherein R_b is -(C_2-C_8)alkyl-N(R_g)(R_h). Another specific value for R_3 is -O-C(=O)N$R_d$$R_e$, wherein R_d is -(C_1-C_8)alkyl-R_f.

A specific value for R_b is -(C_5-C_8)alkyl-N(R_g)(R_h). Another specific value for R_b is -$(CH_2)_6$-N(R_g)(R_h).

A specific value for R_d is -(C_1-C_3)alkyl-R_f. Another specific value for R_d is -CH_2-R_f.

A specific value for R_f is 2-pyridyl.

A sprcific value for R_4 is hydrogen. Another specific value for R_4 is methyl.

A specific value for R_5 is methyl.

A specific value for A^- is halo (e.g. chloro or bromo).

A specific group of compounds are compounds of formula I wherein R_3 is -OP(=O)(OR_a)OR_b; R_a is hydrogen; R_b is -(C_2-C_8)alkyl-N(R_g)(R_h); and R_g, and R_h are each independently (C_1-C_3)alkyl; or a pharmaceutically acceptable salt thereof.

Another specific group of compounds are compounds of formula I wherein R_3 is -O-C(=O)NR_dR_e; R_d is -(C_1-C_8)alkyl-R_f; R_e is (C_1-C_6)alkanoyl; R_f is 2-pyridyl, 3-pyridyl, or 4-pyridyl, substituted at the 1-position with (C_1-C_6)alkyl to form an ammonium salt; or a pharmaceutically acceptable salt thereof.

A preferred group of compounds are compounds of formula I which are quaternary ammonium salts of the nitrogen of R_b, R_c, R_d, or R_f.

A preferred salt of a compound of formula I is a salt wherein R_b, R_c, or R_d is -(C_2-C_8)alkyl-N^+(R_g)(R_h)(R_j) A^-; wherein R_j is (C_1-C_3)alkyl; and A^- is a pharmaceutically acceptable anion.

Another preferred salt of a compound of formula I is a salt wherein R_f is 2-pyridyl, 3-pyridyl, or 4-pyridyl, substituted at the 1-position with (C_1-C_6)alkyl.

Another preferred salt of a compound of formula I is a salt wherein R_3 is -OP(=O)(OR_a)OR_b; R_a is hydrogen; R_b is -(C_2-C_8)alkyl-N^+(R_g)(R_h)(R_j) $^-$A; R_g, R_h, and R_j are each independently (C_1-C_3)alkyl; and $^-$A is a pharmaceutically acceptable anion.

Another preferred salt of a compound of formula I is a salt wherein R_3 is -O-C(=O)NR_dR_e; R_d is -(C_1-C_8)alkyl-R_f; R_e is (C_1-C_6)alkanoyl; R_f is 2-pyridyl, 3-pyridyl, or 4-pyridyl, substituted at the 1-position with (C_1-C_6)alkyl to form an ammonium salt.

A specific group of compounds are compounds of formula II wherein R_6 is benzyl or phenethyl, optionally substituted by 1, 2, or 3 halo, hydroxy, (C_1-C_6)alkyl, (C_1-C_6)alkoxy, (C_1-C_6)alkanoyl, (C_1-C_6)alkanoyloxy, trifluoromethyl, trifluoromethoxy, nitro, cyano, or amino.

Another specific group of compounds are compounds of formula II wherein R_6 is phenethyl, optionally substituted with (C_1-C_6)alkyl.

Another specific group of compounds are compounds of formula II wherein R_6 is 4-isobutylphenethyl.

A compound of formula I can conveniently be prepared from glycerol using techniques which are known in the art.

A compound of formula II can conveniently be prepared using procedures similar to those used to prepare Y-24180 or WEB 2086.

Compounds of formula I or II are also useful as intermediates to prepare other compounds of formula I or II, respectively. For example, compounds of formula I wherein R_b, R_c, or R_d is -(C_2-C_8)alkyl-N(R_g)(R_h) can be alkylated with an alkyl halide (R_j-A) to give a salt of formula I wherein R_b, R_c, or R_d is -(C_2-C_8)alkyl-N^+(R_g)(R_h)(R_j) A^-. Additionally, compounds of formula I wherein R_f is 2-pyridyl, 3-pyridyl, or 4-pyridyl can be alkylated to give a compound of formula I wherein R_f is 2-pyridyl, 3-pyridyl, or 4-pyridyl, substituted at the 1-position with (C_1-C_6)alkyl.

In cases where compounds are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compounds as salts may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids which form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Pharmaceutically acceptable salts may be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids can also be made. Compounds of formula I or II may comprise more than one acidic or basic site capable of salt formation. Accordingly, the pharmaceutically acceptable salts of the invention include mono, di, tri, etc. salts of compounds of formula I or II.

Identification of Agents Falling Within the Scope of the Invention

Therapeutic agents for use in the methods of the invention are those that inhibit the cytokine, growth factor and/or immunoglobulin mediated pathogenic activity of eosinophils. Preferred therapeutic agents for use in the methods of the invention are those that inhibit cytokine, e.g., IL-5, induced degranulation of eosinophils, e.g., PAF receptor antagonists, as well as derivatives or analogs thereof. *In vitro* methods for determining whether a particular agent is within the scope of the invention include, but are not limited to, those provided below in Example 1. Other *in vitro* methods may also be employed, such as methods to detect or determine the effect of the agent on MBP, ECP or EPO release from eosinophils, or LTC4 or cytokine production (Kita et al., 1995) from eosinophils. Also, the efficacy of the agent on eosinophils can be measured in the presence of a moiety that inhibits eosinophil adherence, e.g., anti-CD18, to determine whether the agent inhibits adherence-independent degranulation of eosinophils.

Animal models for a particular disorder or disease may also be employed to screen for therapeutic agents useful in the practice of the methods of the invention. For example, a murine model of the allergen-induced IgE-mediated eosinophil-rich late phase reaction induced by immunization of BALB/c mice with ragweed extract is available (Kaneko et al., Int. Arch. Allergy Appl. Immunol., 96, 41 (1991)). In this model, mice are sensitized by a series of five injections of a ragweed pollen extract and on day 20 are challenged by an intraperitoneal injection of 0.2 ml of a 1:1,000 dilution of ragweed pollen extract (Greer Laboratories, Inc., Lenoir, NC). Agents that inhibit platelet activating factor can be administered prior to, concurrently, and/or subsequent to ragweed pollen extract administration. Forty-eight hours after challenge, peritoneal lavage fluids are collected, and the numbers and kinds of cells determined by total and differential leukocyte counts (after staining of the peritoneal cells with May-Grunwald-Giemsa stain). With this model, in the absence of agent administration, marked eosinophilia occurs at 48 hours and is associated with eosinophil degranulation. RT-PCR analyses have shown that peritoneal cells express IL-5. In addition, eosinophil degranulation can be estimated by measuring the amount of cationic granule proteins, e.g., EPO

activity using o-phenylendiamine as substrate, in peritoneal fluids. Thus, for example, to test the effectiveness of CV6209 on the allergen-specific late phase reaction, CV6209 is administered by intraperitoneal injection 6 hours prior to allergen challenge. For example, a suitable dose of CV6209 can include about 0.01 to about 10 mg/kg, preferably about 0.1 to about 1 mg/kg, although other dosages may be efficacious.

Pulmonary late phase reactions may also be used, because of the ability to administer CV6209 to the lung by local instillation in such reactions. For the experiments described above, it may be desirable to give large doses of agents of the invention. If the desired concentrations of drugs with the peritoneal model of the late phase reaction cannot be achieved, the same reagents can be employed to sensitize mice by pulmonary challenge and elicitation of BAL eosinophilia and eosinophil degranulation.

Other animal models useful to assay the agents of the invention include guinea pigs, mice, and transgenic mice that overexpress the PAF receptor. One of the signal abnormalities in bronchial asthma is bronchial hyperactivity. Bronchial hyperactivity is manifested in patients as a marked irritability of the respiratory tract to nonspecific stimuli including cold air, dust, and, in the laboratory, to inhaled methacholine. Indeed, this hyperactivity is a diagnostic criterion for asthma (N.J. Gross et al., in Allergy, Principles and Practice, Vol. I., E4 Middleton, Jr. et al., eds. (1988) at page 790). Guinea pigs can be sensitized with ovalbumin by nebulization, and bronchial hyperreactivity assessed by administration of aerosolized acetylcholine. Following sensitization and challenge of guinea pigs with ovalbumin, a significant increase in the number of eosinophils are present in the BAL fluids at 24 hours, and at 72 hours after ovalbumin inhalation, bronchial activity to aerosolized acetylcholine is increased, as shown by a significant decrease in the concentrations needed to cause changes in lung resistance and dynamic compliance. Guinea pigs administered an agent of the invention show suppressed bronchial hyperreactivity to aerosolized acetylcholine, and BAL eosinophilia. Thus, therapeutic interventions designed specifically to inhibit PAF may inhibit alterations in bronchopulmonary function.

Other measures of agent efficacy can include the measurement of forced expiratory volume ($FEV_{1)}$ or peak expiratory flow rate (PFR), a decrease in steroid amount or use, inhibition of an exudate of eosinophils, degenerating bronchial epithelial cells, Creola bodies and Charcot-Leyden crystals in the bronchial lumen. In the bronchial mucosa and submuscoa, efficacy can be measured by the inhibition of edema, separation and shedding of ciliated cells, and eosinophil infiltration. In the subepithelial region, the agent may inhibit or reduce the thickening of the basement membrane and eosinophil infiltration, enlargement of glands and smooth muscle hypertrophy.

Targeting of the Therapeutic Agent

Therapeutic agents of the invention, e.g., CV6209, an analog or a derivative thereof, may be targeted to a specific therapeutic site by linking the therapeutic agent to a moiety that specifically binds to a cellular component, e.g., antibodies or fragments thereof, and small molecule drugs, so as to form a therapeutic conjugate. Minimal peptides, mimetic organic compounds and human or humanized antibodies that localize to a specific therapeutic site are also useful as binding peptides of the present invention. Such binding peptides may be identified and constructed or isolated in accordance with known techniques. Preferred binding peptides of these embodiments of the present invention bind to a target epitope with an association constant of at least about 10^{-6} M.

Targeting of the therapeutic agents of the invention can result in increased concentration of the therapeutic agent at a specific anatomic location or to a specific cell type, i.e., eosinophils. Moreover, the linking of a therapeutic agent of the invention to a binding moiety to form a conjugate may increase the stability of the therapeutic agent *in vivo*. Thus, for example, to target eosinophils, eosinophil-binding proteins, e.g., polypeptides or carbohydrates, proteoglycans and the like, that are associated with the cell membranes of eosinophils can be employed to prepare therapeutic conjugates. Likewise, to treat or prevent asthma, antibodies to the bronchial epithelial or to eosinophils may be useful to prepare immunoconjugates for use in the methods of the invention. Such targeting agents include, but are not limited to, the IL-5 receptor, or the α or β chain of the IL-5 receptor, or a fragment thereof which

specifically binds to eosinophils. The α chain of the IL-5 receptor, or a fragment thereof, is a preferred targeting agent of the invention.

To prepare conjugates useful for targeting eosinophils, an antibody or fragment thereof, or other targeting moiety, having a specificity for a surface antigen on eosinophils is attached to a therapeutic agent of the invention. The attachment is via a linker that is covalently attached to both the antibody and the therapeutic agent. More preferably, the linker is a peptide which has a proteolytic cleavage site. This site, interposed between the targeting moiety, e.g., immunoglobulin, and the agent, can be designed to provide for proteolytic release of the agent at, or more preferably, within the eosinophil. For example, it is well known that plasmin and trypsin cleave after lysine and arginine residues at sites that are accessible to the proteases. Many other site-specific endoproteases and the amino acid sequences they attack are well known.

The antigen binding activity of the purified immunoconjugates can then be measured by methods well known to the art, such as described in Gillies et al. (J. Immunol. Methol., 125, 191 (1989)). For example, immunoconjugate activity can be determined using antigen-coated plates in either a direct binding or competition assay format. In particular, it is preferred that humanized antibodies are prepared and then assayed for their ability to bind antigen. Methods to determine the ability of the humanized antibodies to bind antigen may be accomplished by any of numerous known methods for assaying antigen-antibody affinity.

Humanized antibodies (or fragments thereof) are useful tools in methods for therapeutic purposes. When determining the criteria for employing humanized antibodies or antibody conjugates for *in vivo* administration for therapeutic purposes, it is desirable that the general attainable targeting ratio is high and that the absolute dose of therapeutic agent delivered to the target tissue or cell is sufficient to elicit a significant therapeutic response. Methods for utilizing the humanized antibodies can be found, for example, in U.S. Patent Nos. 4,877,868, 5,175,343, 5,213,787, 5,120,526, and 5,202,169. It will be recognized that the inventors also contemplate the utility of human monoclonal antibodies or "humanized" murine antibody as a eosinophil binding protein in the therapeutic conjugates of their invention. For example, murine monoclonal

antibody may be "chimerized" by genetically recombining the nucleotide sequence encoding the murine Fv region (i.e., containing the antigen binding sites) with the nucleotide sequence encoding a human constant domain region and an Fc region, e.g., in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Humanized eosinophil binding partners will be recognized to have the advantage of decreasing the immunoreactivity of the antibody or polypeptide in the host recipient, which may thereby be useful for increasing the *in vivo* half-life and reducing the possibility of adverse immune reactions. See also, N. Lonberg et al. (U.S. Patent Nos. 5,625,126; 5,545,806; and 5,569,825); and Surani et al. (U.S. Patent No. 5,545,807).

Methods useful to prepare antibody-peptide conjugates are well known to the art. See, for example U.S. Patent No. 5,650,150, the disclosure of which is incorporated by reference herein. Representative "coupling" methods for linking the therapeutic agent through covalent or non-covalent bonds to the targeting moiety include chemical cross-linkers and heterobifunctional cross-linking compounds (i.e., "linkers") that react to form a bond between reactive groups (such as hydroxyl, amino, amido, or sulfhydryl groups) in a therapeutic agent and other reactive groups (of a similar nature) in the targeting moiety. This bond may be, for example, a peptide bond, disulfide bond, thioester bond, amide bond, thioether bond, and the like. In one illustrative example, conjugates of monoclonal antibodies with drugs have been summarized by Morgan and Foon (Monoclonal Antibody Therapy to Cancer: Preclinical Models and Investigations, Basic and Clinical Tumor Immunology, *Vol. 2*, Kluwer Academic Publishers, Hingham, MA) and by Uhr, J. of Immunol. 133:i-vii, 1984). In another illustrative example where the conjugate contains a radionuclide cytostatic agent, U.S. Patent No. 4,897,255, Fritzberg et al., incorporated herein by reference, is instructive of coupling methods that may be useful.

One skilled in the art, based on the teachings herein and the applications referenced herein, can readily determine an effective therapeutic effective dosage and treatment protocol. This will depend upon factors such as the particular selected therapeutic agent, route of delivery, the type of target site(s), affinity of the targeting moiety for target site of interest, any cross-

reactivity of the targeting moiety with normal tissue, condition of the patient, whether the treatment is effected alone or in combination with other treatments, among other factors.

For example, a suitable dosage ranges from about 0.001 to about 10 mg, and more preferably from about 0.1 to 2 mg, may be employed.

The conjugates may be administered by known methods of administration. Known methods of administration include, by way of example, intraperitoneal injection, intravenous injection, intramuscular injection, intranasal administration, among others. Intravenous administration is generally preferred.

Dosages, Formulations and Routes of Administration of the Agents of the Invention

The agents of the invention may be administered locally or systemically. While it is possible that for use in therapy the PAF receptor antagonist (the "active agent") or their salts may be administered as the pure dry chemicals, as by inhalation of a fine powder via an insufflator, it is preferable to present the active ingredient as a pharmaceutical formulation. The invention provides a pharmaceutical formulation comprising one or more active agent, or pharmaceutically acceptable salts thereof, together with one or more pharmaceutically acceptable carriers therefor and, optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be 'acceptable' in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Pharmaceutical formulations include those suitable for oral or parenteral (including intramuscular, subcutaneous and intravenous) administration. Forms suitable for parenteral administration also include forms suitable for administration by inhalation or insufflation or for nasal, or topical (including buccal, rectal, vaginal and sublingual) administration. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active compound with liquid carriers, solid matrices, semi-solid carriers, finely

divided solid carriers or combinations thereof, and then, if necessary, shaping the product into the desired delivery system.

Pharmaceutical formulations suitable for oral administration may be presented as discrete unit dosage forms such as hard or soft gelatin capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or as granules; as a solution, a suspension or as an emulsion; or in a chewable base such as a synthetic resin or chicle. The active ingredient may also be presented as a bolus, electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets may be coated according to methods well known in the art, i.e., with enteric coatings.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives.

The compounds according to the invention may also be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

For topical administration to the epidermis, the compounds may be formulated as ointments, creams or lotions, or as the active ingredient of a transdermal patch. Suitable transdermal delivery systems are disclosed, for example, in A. Fisher et al. (U.S. Patent No. 4,788,603), or R. Bawa et al. (U.S. Patent Nos. 4,931,279; 4,668,506 and 4,713,224). Ointments and creams may,

for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents. The active ingredient can also be delivered via iontophoresis, e.g., as disclosed in U.S. Patent Nos. 4,140,122; 4,383,529; or 4,051,842.

When desired, the above-described formulations can be adapted to give sustained release of the active ingredient employed, e.g., by combination with certain hydrophilic polymer matrices, e.g., comprising natural gels, synthetic polymer gels or mixtures thereof.

Pharmaceutical formulations suitable for rectal administration wherein the carrier is a solid are most preferably presented as unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art, and the suppositories may be conveniently formed by admixture of the active compound with the softened or melted carrier(s) followed by chilling and shaping in molds.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or sprays containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

Pharmaceutical formulations include those suitable for administration by inhalation or insufflation or for nasal, intraocular or other topical (including buccal and sub-lingual) administration. The formulations may, where appropriate, be conveniently presented in discrete dosage units and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association the active compound with liquid carriers or finely divided solid carriers or both.

For administration to the upper (nasal) or lower respiratory tract by inhalation, the compounds according to the invention are conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane,

dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the composition may take the form of a dry powder, for example, a powder mix of the active agent and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, e.g., gelatine or blister packs from which the powder may be administered with the aid of an inhalator, insufflator or a metered-dose inhaler.

For intra-nasal administration, the active agent may be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Wintrop) and the Medihaler (Riker).

Drops, such as eye drops or nose drops, may be formulated with an aqueous or nonaqueous based also comprising one or more dispersing agents, solubilizing agents or suspending agents. Liquid sprays are conveniently delivered from pressurized packs. Drops can be delivered via a simple eye dropper-capped bottle, or via a plastic bottle adapted to deliver liquid contents dropwise, via a specially shaped closure.

Formulations suitable for topical administration in the mouth or throat include lozenges comprising active agent in a flavored base, usually sucrose and acacia or tragacanth; pastille comprising the active agent in an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the active agent in a suitable liquid carrier.

The pharmaceutical compositions according to the invention may also contain other active ingredients such as antimicrobial agents, or preservatives. The active agent may also be used in combination with other therapeutic agents, for example, bronchodilators or anti-inflammatory agents.

It will be further appreciated that the amount of the active agent required for use in treatment will vary not only with the particular compound selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at

the discretion of the attendant physician or veterinarian. In general, however, a suitable unit dose for counteracting respiratory tract symptomatology will deliver per day from about 0.001 to about 100 mg/kg, preferably about 0.01 to about 10 mg/kg, of body weight.

Useful dosages of the agents of the invention can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Patent No. 4,938,949.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two-, three-, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye or nose.

The invention will be further described by reference to the following detailed examples.

Example 1

Attenuation of Eosinophil Activation by Inhibiting Autocrine Production of PAF

Materials and Methods

Reagents. Platelet activating factor (PAF) and CV6209 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA) and were dissolved in absolute ethanol at a concentration of 40 mM and stored at -20°C. Phorbol myristate acetate (PMA) was purchased from Calbiochem (La Jolla, CA), dissolved with dimethyl sulfoxide at a concentration of 5 mg/ml and stored at -20°C. Human IL-5 was a generous gift from Schering-Plough Research Institute (Kenilworth, NJ) and was diluted with phosphate-buffered saline containing 0.1% bovine serum albumin to 100 μg/ml and was stored at -70°C. Mepacrine and anti-CD18 were purchased from Sigma Chemical Co. (St. Louis, MO) and Becton Dickinson (San Jose, CA), respectively.

All agonists and antagonists were diluted in reaction medium immediately before the experiments. Purified human serum IgG was purchased from Organon Teknika-Cappel (Malvern, PA) and was stored at 1 mg/ml in PBS at 4°C. For eosinophil activation, IgG was diluted to 50 mg/ml with PBS and 50

µl per well added to plates for overnight incubation at 4°C. Catalase, superoxide dismutase, human serum albumin (HSA), taurine and cytochrome c were purchased from Sigma Chemical Co. Pertussis toxin (PTX) was purchased from Calbiochem.

Eosinophil isolation. Eosinophil isolation was performed with minor modifications of the method described by Ide et al. by a magnetic cell separation system (MACS; Becton Dickinson, San Jose, CA). Briefly, venous blood anticoagulated with 50 U/ml heparin was obtained from normal volunteers and diluted with PBS at a 1:1 ratio. Diluted blood was overlaid on isotonic Percoll solution (density, 1.085 g/ml; Sigma Chemical Co.) and centrifuged at 1000 *g* for 30 minutes at 4°C. The supernatant and mononuclear cells at the interface were carefully removed, and erythrocytes in sediment were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with piperazine-N,N'bis[2-ethanesulfonic acid] (Pipes) buffer (25 mM Pipes, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4) with 1% fetal calf serum (FCS) (HyClone Laboratories; Logan, UT), and an approximately equal volume of anti-CD16-conjugated magnetic beads (Miltenyi Biotech Inc.; Auburn, CA) was added to the cell pellet. After 60 minutes of incubation on ice, cells were loaded onto the separation column positioned in the strong magnetic filed of the MACS. Cells were eluted three times with 5 ml of Pipes buffer with FCS. The purity of eosinophils counted by Randolph's stain was greater than 94%. Purified eosinophils were washed and suspended in reaction medium, then tested immediately.

Superoxide anion production. Eosinophil superoxide production was induced by various stimuli in polystyrene 96-well flat-bottom tissue culture plates (Falcon #3072, Becton Dickinson). To immobilize IgG onto the wells of the plate, selected wells were coated overnight at 4°C with 50 µl of IgG dissolved in PBS at 50 µg/ml. The solution was aspirated; all wells were coated with 50 µl of 2.5% HSA (Sigma, A3782) dissolved in PBS and incubated at 37°C for 2 hours. After incubation, wells were washed twice with 0.9% NaCl and used immediately for the experiments. Generation of superoxide by eosinophils was measured by reduction of cytochrome c, as described previously (Sedgwick et al., 1988), with slight modifications.

Freshly isolated eosinophils were washed with Hanks' balanced salt solution (HBSS) with 10 mM HEPES and resuspended to 5×10^5 cells/ml in cytochrome c (Sigma) solution (2.4 mg/ml cytochrome c in HBSS medium). Fifty µl of antagonist (CV6209 or mepacrine) at 4X concentrated stock (the final concentration) or medium was added to each well. One hundred microliters of cell suspension was dispensed onto plates and the reactions were initiated by adding 50 µl of soluble stimulus diluted in HBSS with 10 mM HEPES. Wells coated with immobilized Ig received 50 µl of HBSS with 10 mM HEPES. Immediately after addition of stimuli, the reaction wells were measured for absorbance at 550 nm in a microplate autoreader (Thermomax, Molecular Devices; Menlo Park, CA), followed by repeated readings. Between absorbance measurements, the plate was incubated at 37°C. Superoxide anion generation was calculated using the following equation: 19.1 (absorbance - $\text{absorbance}_{\text{time0}}$)/0.5 = nanomole superoxide produced/10^5 cells (Horie et al., 1996).

Cell stimulation for PAF production. Eosinophils were stimulated to produce PAF using a modification of the method described by van der Bruggen et al. (1994). Generation of PAF by eosinophils was performed in 96-well flat-bottom tissue culture plates, prepared with immobilized Ig and 2.5% HSA as described previously. All incubations were performed in enriched HEPES medium (HEPES supplemented with 1 mM $CaCl_2$, 5 mM glucose, and 0.5% (w/v) HSA) containing O_2-radical scavengers (2.5 mM taurine, 5000 U/ml catalase, and 380 U/ml superoxide dismutase) to prevent lipid degradation. Freshly isolated eosinophils were suspended in enriched HBSS with scavengers at 5 X 10^3 cells/ml; and 100 µl aliquots were added to prepared tissue culture plates. Enriched HBSS with scavengers or IL-5 diluted in enriched HBSS with scavengers was added. Plates were incubated at 37°C for 15 or 45 minutes and centrifuged at 400 *g* for 3 minutes to pellet cells. Aliquots of supernatant were removed and stored under nitrogen at -70°C. PAF was determined with a competitive radioimmunoassay (DuPont NEN, Boston, MA) following the manufacturer's protocol.

Eosinophil degranulation. Eosinophil degranulation was performed in 96-well flat-bottom tissue culture plates, prepared with

immobilized IgG and 2.5% HSA as described previously. Eosinophils were washed with RPMI-1640 medium (Celox Inc., Hopkins, MN) with 10 mM HEPES and 0.02% HSA, and resuspended in the same medium at 5 x 10^5 cells/ml. The soluble stimuli (IL-5, PMA and PAF) and the agonist CV6209 were diluted in the same medium. Fifty microliters of 4-fold concentrated solutions of CV6209 or medium were added to wells, followed by 100 µl of cell suspension. Wells that had been pre-coated with IgG and 2.5% HSA received 50 µl of medium, while wells that had only the 2.5% HSA coating received 50 µl of the appropriate soluble stimuli. Plates were incubated at 37°C and 5% CO_2. In degranulation experiments, plates were removed after 180 minutes of incubation and aliquots of supernatant were removed and stored at -20°C until assayed for eosinophil-derived neurotoxin (EDN) as described below.

EDN radioimmunoassay. To quantitate eosinophil degranulation, concentrations of EDN in the sample supernatants and lysates were measured by radioimmunoassay. The radioimmunoassay is a double-antibody competition assay in which radioiodinated EDN, rabbit anti-EDN and burro anti-rabbit IgG are used as reported elsewhere (Abu-Ghazaleh et al., 1989). Total cellular EDN contents were measured simultaneously using supernatants from cells lysed with 0.5% Nonidet P-40 detergent. All assays were carried out in duplicate.

Statistical analysis. Data are presented as mean (SEM from the numbers of experiments indicated). Statistical significance of the differences between various treatment groups (i.e., with or without inhibitor) was assessed using the Mann-Whitney U test or paired Student's t-test.

Results

Blocking of phospholipase A_2 inhibits IL-5- or immobilized-IgG-induced eosinophil release of superoxide anion. White et al. (1993) have shown that eosinophil superoxide release induced by a combination of fMLP and cytochalasin B is inhibited by mepacrine, a non-specific inhibitor of PLA_2 that functions by direct enzyme interaction (Mahadevappa et al., 1990; Jain et al., 1991). To determine the effects of PLA_2 inhibition on IL-5- and IgG-mediated superoxide production, eosinophils were stimulated with IL-5 (25 ng/ml), PMA

(1 ng/ml) or immobilized-IgG (50 μg/ml) along with increasing concentrations of mepacrine.

As shown in Figure 1, mepacrine inhibited IgG-induced superoxide production and IL-5-induced superoxide production in a concentration-dependent manner. In contrast, PMA-induced superoxide production was not affected by the addition of up to 100 μM mepacrine. Thus, PLA_2 is likely involved in superoxide production by eosinophils stimulated with immobilized IgG or IL-5.

Agonist-stimulated eosinophils release PAF in a time-dependent manner. Because PLA_2 is involved in the metabolism of arachidonic acid, it was determined whether PAF is produced when eosinophils are stimulated with soluble IL-5, immobilized-IgG or PMA. As a result, human eosinophils released PAF in response to IL-5 and immobilized IgG in a time-dependent manner (Figure 2). PAF was detectable 15 minutes after stimulation with immobilized IgG or soluble PMA. After 45 minutes, PAF was also detectable in supernatants from eosinophils stimulated with IL-5. Eosinophils responded more vigorously to immobilized IgG (2.9 ± 0.4 ng PAF/10^6 cells) and PMA (3.6 ± 1.0 ng PAF/10^6 cells), but less vigorously to IL-5 (0.5 ± 0.1 ng PAF/10^6 cells). At 45 minutes, all three stimuli induced significant release of PAF, $p < 0.05$ compared to medium alone (MWU). Eosinophils incubated in medium alone did not release detectable levels of PAF at any time.

To confirm whether PAF release induced by agonists involves PLA_2, eosinophils were stimulated with IL-5 and immobilized-IgG in the presence of mepacrine, an inhibitor of PLA_2. The IL-5- or immobilized IgG-induced release of PAF was blocked by $\geq$ 60% by 30 μM mepacrine.

CV6209 is a competitive antagonist for the PAF receptor. CV6209 is known as a potent PAF receptor antagonist (Terashita et al., 1987). When eosinophils were stimulated with PAF at concentrations ranging from 0.1 to 3 μM, PAF induced superoxide production in a concentration-dependent manner (Figure 3). At each concentration of PAF, as the concentration of CV6209 was increased, superoxide release dropped. CV6209 at 0.1 μM blocked 60% of the superoxide released from eosinophils stimulated with 0.1 but had little or no effect on superoxide release from eosinophils incubated with 0.3, 1 or

3 μM PAF. Increasing the concentration of CV6209 to 0.3 μM resulted in 93% and 62% decreases in superoxide released from cells stimulated with 0.1 to 0.3 μM PAF, respectively, but had little effect when the PAF was 1 or 3 μM. Taken together, these results indicate that CV6209 competitively inhibits PAF-induced superoxide release by eosinophils.

PAF is required for eosinophil superoxide production. As shown previously (Kaneko et al., 1995; Kroegel et al., 1989; Horie et al., 1994; Sedgwick et al., 1988), stimulation of eosinophils with immobilized IgG, PAF, IL-5 or PMA induced superoxide production in a time-dependent manner (Figure 4). At 30 minutes and later, increasing concentrations of CV6209 inhibited the release of superoxide from eosinophils stimulated with all agonists except PMA. CV6209 produced no effect on unstimulated eosinophils. IL-5- and PAF-induced eosinophils appeared equally sensitive to CV6209, with an ED_{50} of approximately 0.3 μM. Immobilized-IgG-induced eosinophils appeared to be less sensitive, with an ED_{50} of approximately 1 μM. After stimulation with PMA, eosinophils showed a minimal response to CV6209. After 180 minutes of incubation, 0.1, 0.3 and 1 μM CV6209 significantly inhibited immobilized IgG- and soluble PAF-induced superoxide ($p < 0.05$ for all concentrations with both agonists) (Figure 5). Moreover, 0.1 μM CV6209 caused a 19.2% reduction of superoxide release from cells stimulated with IL-5 ($p = 0.059$); 0.3 and 1 μM concentrations reduced superoxide release by 49.8% ($p < 0.05$) and 90.4% ($p < 0.05$) respectively. After stimulation with PMA, eosinophils showed a minimal response to CV6209.

Effects of other selected PAF receptor antagonists. To confirm the results of experiments with CV6209, the effects of other PAF receptor antagonists with different structures were examined, namely hexanolamine PAF and Y24180 (Grigoriadis et al., 1991; Tokumura et al., 1986; Takehana et al., 1990). As shown in Figure 8, eosinophil superoxide production stimulated with IL-5 was inhibited by both reagents in a concentration-dependent manner. These findings support the hypothesis that PAF receptor antagonists potently inhibit IL-5 induced activation of eosinophils.

PAF is required for eosinophil degranulation stimulated by immobilized immunoglobulins, IL-5 and PAF. To investigate the role of

autocrine PAF for degranulation of eosinophils, purified cells were stimulated with immobilized IgG or soluble IL-5 and the effects of CV6209 were examined. By 180 minutes of incubation, both agonists stimulated eosinophils to release EDN (Figure 6, solid bars). Addition of 0.3 μM CV6209 reduced EDN release from eosinophils stimulated with IL-5 by 15% ($p < 0.05$) (Figure 6, grey bars). Addition of 0.3 μM CV6209 reduced EDN release from eosinophils stimulated with immobilized IgG by 13%; this change was not significant. When the concentration of CV6209 was increased to 1 μM, degranulation was reduced by 49% in immobilized IgG-stimulated cells and by 35% in IL-5 stimulated cells ($p < 0.05$, $p = 0.05$, respectively) (Figure 6, open bars). Thus, autocrine PAF is likely involved, at least in part, in the degranulation of eosinophils stimulated with immobilized IgG or IL-5. In contrast, additional experiments showed two other PAF receptor antagonists, WEB 2086 and SM-10661, did not inhibit IL-5- and immobilized IgG-induced degranulation at concentrations of 0.3 μM.

Cellular adhesion is required for PAF production, eosinophil degranulation and superoxide production. Cellular adhesion mediated through β_2-integrins plays a critical role in eosinophil effector functions (Horie et al., 1994). To determine the effects of eosinophil adhesion on the IL-5- or immobilized-IgG-induced release of PAF, eosinophils were incubated with an anti-CD18 or with a mouse isotype control IgG_1 antibody prior to eosinophil activation. Mouse isotype control antibody had no effect on PAF release by stimulated eosinophils. However, when adhesion was blocked by anti-CD18, release of PAF was significantly inhibited (Table 1, $p < 0.05$ compared to $mIgG_1$, for both immobilized IgG and soluble IL-5).

Table 1

	Agonist	Agonist alone	With αCD18	With $mIgG_1$
PAF Release $ng/10^6$ cells	Medium	0.15 ± 0.03		
	IgG	2.91 ± 0.49	0.41 ± 0.21†	4.09 ± 1.68
	IL-5	0.53 ± 0.15	0.28 ± 0.11†	0.68 ± 0.21
Degranulation (Released EDN, ng/ml)	Medium	0‡	0‡	
	IgG	34.0 ± 3.9	4.2 ± 3.1*	
	IL-5	12.8 ± 1.0	2.0 ± 1.5*	
	PAF	12.4 ± 3.3	1.3 ± 1.4	
Superoxide Production ($nmole/10^5$ cells)	Medium	-1.21 ± 0.84	-1.05 ± 0.78	
	IgG	13.87 ± 1.90	3.71 ± 1.37*	
	IL-5	11.02 ± 1.41	0.06 ± 0.03*	
	PAF	7.45 ± 2.07	-0.21 ± 0.59*	

Data are presented as means ± SEM of four experiments (PAF release), three experiments (degranulation) or five experiments (superoxide).

* $p < 0.05$ compared to agonist alone
† $p < 0.05$ compared to agonist plus $mIgG_1$
‡ Background levels (medium) have been subtracted

To determine the effects of eosinophil adhesion on degranulation, purified eosinophils were incubated with anti-CD18 prior to stimulation with immobilized IgG or soluble IL-5, or PAF. After 90 minutes, the IgG-stimulated eosinophils released significantly lower levels of EDN than eosinophils incubated without antibody (88% decrease, $p < 0.05$) (Table 1). Likewise, the IL-5-stimulated eosinophils released significantly lower levels of EDN than eosinophils incubated without antibody (84% decrease, $p < 0.05$). Although eosinophils stimulated with PAF in the presence of anti-CD-18 released markedly less EDN than those incubated with PAF alone (89%), this drop was not statistically significant.

To determine the effects of eosinophil adhesion on superoxide production, purified eosinophils were incubated with anti-CD18 prior to stimulation with immobilized IgG or soluble IL-5, PMA or PAF. After 180

minutes, IgG-, IL-5- and PAF-stimulated eosinophils incubated with anti-CD18 released significantly lower levels of superoxide than eosinophils incubated without antibody (73% decrease, $p < 0.05$; 80% decrease, $p < 0.05$; 100% decrease, $p < 0.05$, respectively). Eosinophils stimulated with PMA responded similarly to the stimuli whether or not anti-CD18 was included. Overall, these three experiments suggest that β_2-integrin-dependent adhesion is necessary for IL-5-, PAF- or immobilized IgG-induced eosinophil release of PAF, and superoxide anion and EDN production (degranulation).

IL-5-induced superoxide production is PTX sensitive. The receptor for IL-5 belongs to the hemopoietin receptor family, which has not been shown to be coupled to G-proteins. In contrast, the PAF receptor is coupled to G-proteins (Houslay et al., 1986; Hwang et al., 1986; Uhing et al., 1989). PTX catalyzes the ADP-ribosylation of certain G-protein α-subunits in intact cells, resulting in the uncoupling of these G-proteins from cell surface receptors (Moss, 1988). To examine the potential involvement of a pertussis toxin (PTX)-sensitive G-protein in activation of eosinophils stimulated with IL-5, purified eosinophils were pre-incubated with PTX for two hours at 37°C and then stimulated with IL-5, PMA or PAF. PTX-treated eosinophils stimulated with IL-5 released 66% less superoxide than did non-pretreated eosinophils (Figure 7, $p < 0.05$). Similarly, PTX-treated eosinophils stimulated with PAF released 94% less superoxide than did non-pretreated eosinophils ($p < 0.05$). In contrast, PTX-treated eosinophils stimulated with PMA released slightly more superoxide than did non-pretreated eosinophils. Thus, PTX-sensitive G-proteins are likely involved in eosinophil activation stimulated by IL-5 as well as by PAF.

CV6209 inhibits LTC4 release by activated eosinophils. Eosinophils were preincubated with various concentrations of CV6209, and then stimulated with IL-5, PAF, or immobilized human IgG for 60 minutes. Supernatants were collected and analyzed by a LTC4 ELISA kit. As shown in Figure 9, LTC4 production and release by eosinophils stimulated with IL-5 and immobilized IgG as well as that stimulated with PAF were inhibited by CV6209 in a concentration-dependent manner. These findings suggest that autocrine PAF is involved in LTC4 production by eosinophils activated by IL-5 or immobilized IgG.

Discussion

IL-5 and IgG effectively activate eosinophils, causing cellular adhesion, degranulation, superoxide anion production and release of lipid mediators such as PAF (Gleich et al., 1994; Lee et al., 1984; Sedgwick et al., 1988; Trigianni et al., 1992; White et al., 1993; Cromwell et al., 1990). PAF itself induces eosinophil activation, specifically adhesion, degranulation and superoxide production (Gleich et al., 1994). Eosinophil growth factors such as IL-5, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are released by activated eosinophils *in vitro* and are known to activate eosinophils themselves, suggesting an autocrine role for these cytokines in eosinophil-associated inflammation (Gleich et al., 1994). Whether PAF acts similarly, in an autocrine fashion, has not been studied. As described above, eosinophils stimulated with immobilized IgG or with soluble IL-5 produced biologically active levels of PAF. Moreover, PAF produced by activated eosinophils enhanced degranulation and superoxide anion production in those same eosinophils and that this auto-upregulation most likely occurs via a G-protein coupled receptor.

Many agonists induce eosinophils to release PAF. For example, calcium ionophores, fMLP, C5a and ECF-A stimulate rapid (1 to 3 minutes) release of PAF from eosinophils (White et al., 1993). IgG coated sepharose beads and soluble GM-CSF also stimulate eosinophils to release PAF (Cromwell et al., 1990; Triggiani et al., 1992). Further, eosinophils stimulated with either immobilized IgG or soluble IL-5 released nM levels of PAF (Figure 2). Release of PAF by immobilized IgG- or soluble IL-5-induced cells was inhibited by mepacrine, a drug that blocks generation of PAF by binding with a phospholipid substrate of PLA_2. This suggests that upon stimulation with immobilized-IgG or soluble IL-5 eosinophils are rapidly producing PAF rather than merely releasing stored PAF.

In earlier reports, PAF itself was shown to stimulate both eosinophil degranulation and production of superoxide (Kroegel et al., 1988; Kroegel et al., 1989). Furthermore, White et al. observed that eosinophil degranulation and superoxide production induced by fMLP plus cytochalasin B can be blocked by the addition of mepacrine (White et al., 1993). In addition,

neutrophil production of superoxide induced by TNF can be inhibited by PAF receptor antagonists. In the same way, monocyte production of superoxide induced by opsonized zymosan can be inhibited by blocking PLA_2 and thus PAF production (Braquet et al., 1991; Li et al., 1997).

It was unknown whether the PAF produced by eosinophils which had been stimulated with soluble IL-5 or immobilized IgG had any effect on eosinophil responses such as superoxide anion production or degranulation. As described above, it was found that superoxide released from eosinophils stimulated with immobilized Ig or soluble IL-5 was inhibited with CV6209, a receptor antagonist, as well as with mepacrine, a PLA_2 inhibitor (Figures 1, 6 and 7). Thus, the generation and release of PAF from activated eosinophils and subsequent re-activation of those eosinophils by the released PAF likely enhances superoxide anion production. Eosinophil release of EDN upon activation with IL-5 or with immobilized IgG was inhibited by a competitive PAF receptor antagonist (Figure 4). Thus, both superoxide production and degranulation in IL-5- and immobilized IgG-activated eosinophils are inhibited when PAF production and receptor binding are inhibited, indicating an autocrine mechanism of activity.

Eosinophils stimulated with PAF degranulate and release EDN and other granule proteins *in vitro*. When PAF-activated cells are prevented from adhering, either by blocking β_2-integrin adhesion using antibody to Mac-1 (CD11b/CD18) or simply by constant stirring, EDN release is blocked (Horie et al., 1994). Eosinophils stimulated with PAF or immobilized IgG released lower levels of EDN when adhesion was blocked with anti-CD18. Release of EDN by IL-5 activated eosinophils appeared to be dependent on β_2-integrin adhesion. When adhesion was blocked with anti-CD, eosinophils stimulated with PAF, IL-5 or immobilized IgG released significantly lower levels of superoxide anion (Table 1). When eosinophils are stimulated with immobilized IgG or soluble IL-5, addition of anti-CD18 significantly inhibits PAF release (Figure 8). These experiments suggest that β_2-integrin adhesion is necessary for PAF-induced degranulation and superoxide production by eosinophils, and that immobilized IgG-induced degranulation, and IL-5- and immobilized-IgG induced superoxide production are also regulated by eosinophil adhesion. Furthermore, PAF

production is dependent on adhesion, indicating that adhesion is necessary not only for eosinophil function (degranulation and superoxide production) but also for the production of PAF, which in turn upregulates degranulation and adhesion.

Previously, treatment of eosinophils with PTX has been shown to completely abolish EDN release from immobilized sIgA-activated eosinophils and to temporarily inhibit EDN release from cells activated with immobilized IgG (Kita et al., 1991). Also, neutrophils primed with PAF and subsequently stimulated with TNF release superoxide in a PTX sensitive manner (Braquet et al., 1991). In general, PAF receptors are thought to signal through a G protein, although whether that response is sensitive to PTX appears to depend on the cell involved (Houslay et al., 1986; Hwang et al., 1986; Uhing et al., 1989). In eosinophils pretreated with PTX, PAF-induced increases in intracellular free calcium were significantly attenuated (Kernen et al., 1991). Eosinophils pretreated with PTX and stimulated with IL-5 or PAF released significantly less superoxide than cells that were not pretreated (Figure 7), suggesting that PTX-sensitive G proteins are involved in eosinophil activation stimulated by IL-5 and PAF. There is no evidence as yet that the IL-5 receptor is coupled to a G protein, and, indeed, structurally such a configuration appears unlikely. For this reason, it appears that IL-5 activates eosinophils not just via the IL-5 receptor, but through another G protein coupled receptor as well, indicating that an IL-5 induced mediator, such as PAF, is necessary.

Earlier studies had suggested an autocrine role for PAF in leukocyte activation. Coeffier et al. showed that IL-5 induced chemotaxis of guinea pig eosinophils was inhibited by the PAF receptor antagonist WEB 2086, indicating that eosinophil migration induced by IL-5 involves, most likely, autocrine PAF (Coeffier et al., 1991). Release of superoxide from neutrophils primed with PAF and stimulated with TNF could be completely blocked with the addition of PAF receptor antagonists; when TNF alone was used as a stimulus, superoxide production was reduced by 25% by PAF receptor antagonists (Braquet et al., 1991). Bo-ting et al. showed that zymosan-induced IL-8 production in neutrophils could be blocked by addition of PAF antagonists (Bo-ting et al., 1994). A recent *in vivo* study showed that ingested SR27417A, a PAF

receptor antagonist, significantly attenuated the late asthmatic response in 12 subjects (Evans et al., 1997).

Upon stimulation with immunoglobulins (IgG) or cytokines (IL-5), eosinophils produce and release PAF. This autocrine PAF stimulates cellular adhesion of eosinophils, which further upregulates PAF production. Together, autocrine PAF and cellular adhesion induce eosinophil effector functions, such as degranulation and superoxide production. Inhibition of either autocrine PAF or adhesion markedly suppresses eosinophil function.

References

Abu-Ghazaleh, R.I., Fuisawa, T., Mestecky, J., Kyle, R.A., Gleich, G.J. IgA-induced eosinophil degranulation. J. Immunol., 142, 2393, 1989.

Allen, J. N. and Davis, W. B. Eosinophilic lung diseases. Am. J. Respir. Crit. Care Med., 150:1423-1438, 1994.

Bo-ting, A., William, T. J., and Collins, P. D. Zymosan-induced IL-8 release from human neutrophils involves activation via the CD11b/CD18 receptor and endogenous platelet-activating factor as an autocrine modulator. J. Immunol., 152:5411-5419, 1994.

Braquet, P., Hosford, D., Koltz, P., Guilbaud, J., and Paubert-Braquet, M. Effect of platelet-activating factor on tumor necrosis factor-induced superoxide generation from human neutrophils: Possible involvement of G proteins. Lipids, 26:1071-1075, 1991.

Broide, D.H., Gleich, G.J., Cuomo, A.J., Coburn, D.A., Federman, E.C., Schwartz, and L.B., Waserman, S.I. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. J. Allergy Clin. Immunol., 88:637-648, 1991.

Butterfield et al., in Antiinflammatory Steroid Action: Basic and Clinical Aspects, Schleimer et al., eds. Academic Press, Inc. (1989).

Chung, K.F. and Barnes, P.J. Review: Role for platelet-activating factor in asthma. Lipids; 26:1277-1279, 1991.

Coeffier, E., Joseph, D., and Vargaftic, B.B. Activation of guinea pig eosinophils by human recombinant IL-5. Select priming to platelet-activating factor- acether and interference of its antagonists. J. Immunol., 147:2595-2602, 1991.

Cromwell, O., Wardlaw, A.J., Champion, A., Moqbel, R., Osei, D., and Kay, A.B. IgG-dependent generation of platelet-activating factor by normal and low density human eosinophils. J. Immunol, 145:3862-3868, 1990.

Evans, D.J., Fryer, A.D., Jacoby, D.B., Gleich, G.J., and Costello, R.W. Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M_2 muscarinic receptors in antigen-challenged guinea pigs. J. Clin. Invest., 100:2254, 1997a.

Evans, D.J., Barnes, P.J., Cluzel, M., and O'Connor, B.J. Effects of a Potent Platelet-activating Factor Antagonist, SR27417A, on Allergen-induced Asthmatic Response. Am J. Respir. Crit. Care Med.. 156:11-16, 1997b.

Filley, W.V., Holley, K.E., Kephart, G.M., Gleich, G.J., Identification by immunoflorescence of eosinophil granule major basic protein in lung tissues of patient with bronchial asthma. Lancet, ii, 11-12, 1982.

Frigas, E. and Gleich, G.J. The eosinophil and the pathophysiology of asthma. J. Allergy Clin. Immunol., 77:527, 1986.

Fujisawa, T., Abu-Ghazaleh, R.I., Kita, H., Sanderson, C.J., Gleich, G.J. Regulatory effect of cytokines on eosinophil degranulation. J. Immunol., 144, 642-646, 1990.

Gleich, G.J. Eosinophil granule proteins and bronchial asthma. Allergol. Int., 45:35, 1996.

Gleich, G.J., Kita, H., Adolphson, C.R., Eosinophils; in Frank, M.M., Austen, K.F., Claman, H.N., Unanue, E.R. (eds); Samter's Immunologic Diseases. Boston, Little, Brown, Vol. 1, 205-245, 1994.

Grigoriadis, G. and Stewart, A.G. 1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho (N, N, N trimethyl) hexanolamine: an analogue of platelet-activating factor with partial agonist activity. Br. J. Pharmacol., 104:171-177, 1991.

Gross et al., in Allergy, Principles and Practice, Vol. I., E4 Middleton, Jr. et al., eds. (1988) at page 790.

Hamid, Q., Azzawi, M., Ying, S., Moqbel, R., Wardlaw, A.J., Corrigan, C.J., Bradley, B., Durham, S.R., Collins, J.V., Jeffery, P.K. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from Asthma Journal of Clinical Investigation, 87, 1541-1546, 1991.

Horie, S., Gleich, G.H., and Kita, H. Cytokines directly induce degranulation and superoxide production from human eosinophils. J. Allergy Clin. Immunol., 98:371-381, 1996.

Horie, S. and Kita, H. CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating factor. J. Immunol., 152:5457-5467, 1994.

Horie, S., Okubo, Y., Hossain, M., Momose, T., Suzuki, J., Isobe, M., and Sekiguchi, M. Intercellular adhesion molecule-1 on eosinophils is involved in eosinophil protein X release induced by cytokines. Immunology, 90:301-307, 1997.

Houslay, M.D., Bojanic, D., and Wilson, A. Platelet activating factor and U44069 stimulate a GTPase activity in human platelets which is distinct from the guanine nucleotide regulatory proteins, Ns and Ni. Biochem. J., 234:737-740, 1986.

Hozawa, S., Haruta, Y., Ishioka, S., and Yamakido. Effects of a PAF antagonist, Y-24180, on bronchial hyperresponsiveness in patients with asthma. Am. J. Respir. Crit. Care Med., 152:1198-202, 1995.

Hwang, S.B., Lam, M.H., and Pong, S.S. Ionic and GTP regulation of binding of platelet-activating factor to receptors and platelet-activating factor-induced activation of GTPase in rabbit platelet membranes. J. Biol. Chem., 261:532-537, 1986.

Ide, M., Weiler, D.A., Kita, H., Gleich, G.J., Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. J. Immunol. Methods, 168, 187-196, 1994.

Ishida, K., Thomson, R.J., Beattie, L.L., Wiggs, B., and Schellenberg, R.R. Inhibition of antigen-induced airway hyperresponsiveness, but not acute hypoxia nor airway eosinophilia, by an antagonist of platelet-activating factor. J. Immunol., 144:3907-3911, 1990.

Ishii, S., Nagase, T., Tashiro, F., Ikuta, K., Sato, S., Waga, I., Kume, K., Miyazaki, J., and Shimizu, T. Bronchial hyperreactivity, increased endotoxin letharity and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. EMBO J., 16:133-142, 1997.

Jain, M.K., Yu, B.Z., Rogers, J., Ranadive, G.N., and Berg, O.G. Interfacial catalysis by phospholipase A_2: Dissociation constants for calcium, substrate, products, and competitive inhibitors. Biochemistry, 30:7306-7317, 1991.

Jain, M.K., Streb, M., Rogers, J., DeHaas, G.H. Action of phospholipase A2 on bilayers containing lysophosphatidylcholine analogs and the effect of inhibitors. Biochemical Pharmacology, 33, 2541-2551, 1984.

Kagoshima, M., Tomomatsu, N., Iwahisa, Y., Yamaguchi, S., Kawakami, Y., and Terasawa, M. Effect of Y-24180, a long-acting antagonist to platelet-activating factor (PAF), on PAF-induced reactions: a relationship between the partial structure of the compound and its duration of the action. Pharmacology, 54(5):261-70, May 1997.

Kagoshima, M., Tomomatsu, N., Iwahisa, Y., Yamaguchi, S., Matsuura, M., Kawakami, Y., and Terasawa, M. Suppressive effects of Y-24180, a receptor antagonist to platelet activating factor (PAF), on antigen-induced asthmatic responses in guinea pigs. Inflammation Research, 46(4):147-53, Apr. 1997.

Kaneko, M., Horie, S., Kato, M., Gleich, G.J., and Kita, H. A crucial role for beta 2 integrin in the activation of eosinophils stimulated by IgG. J. Immunol., 155:2631-2641, 1995.

Kernen, P., Wymann, M.P., von Tscharner, V., Deranleau, D.A., Tai, P.-C., Spry, C.J., Dahinden, C.A., and Baggiolini, M. Shape changes, exocytosis, and cytosolic free calcium changes in stimulated human eosinophils. J. Clin. Invest., 87:2012-2017, 1991.

Kimani, G., Tonnesen, M.G., and Henson, P. Stimulation of eosinophil adherence to human vascular endothelial cells in vitro by platelet-activating factor. J. Immunol., 140:3161-3166, 1988.

Kita, H., Weiler, D.A., Abu-Ghazaleh, R., Sanderson, C.J., Gleich, G.J., Release of granule proteins from eosinophils cultured with IL-5. J. Immunol., 149, 629-635, 1992.

Kita, H., Abu-Ghazaleh, R.I., Sur, S., Gleich, G.J. Eosinophil major basic protein induces degranulation and IL-8 production by human eosinophils. J. Immunol., 154, 4749-4758, 1995.

Kita, H., Abu-Ghazaleh, R.I., Gleich, G.J., and Abraham, R.T. Role of pertussis toxin-sensitive G proteins in stimulus-dependent human eosinophil degranulation. J. Immunol., 147:3466-3473, 1991.

Kroegel, C., Yukawa, T., Dent, G., Venge, P., Chung, K.F., and Barnes, P. J. Stimulation of degranulation from human eosinophils by platelet-activating factor. J. Immunol, 142:3518, 1989.

Krogel, C., Yukawa, T., Dent, G., Chung, K.F., and Barnes, P.J. Platelet activating factor induces eosinophil peroxidase release from human eosinophils. Immunology, 64:559-562, 1988.

Lamas, A., Mulroney, C.M., and Schleimer, R.P. Studies on the adhesive interaction between purified human eosinophils and cultured vascular endothelial cells. J. Immunol., 140:1500-1505, 1988.

Lee, T.-C., Lenihan, D.J., Malone, B., Roddy, L.L., and Wasserman, S.I. Increased biosynthesis of platelet-activating factor in activated human eosinophils. J. Biol. Chem., 259:5526-5530, 1984.

Lefort, T.J., Nahorl, A., Ruffle, C., Vargaftig, B.B., and Pretolani M: *In vivo* neutralization of eosinophil-derived major basic protein inhibits antigen-induced bronchial hyperreactivity in sensitized guinea pigs. J. Clin. Invest., 97:1117, 1996.

Li, Q., and Cathcart, M.K. Selective inhibition of cytosolic phospholipase A_2 in activated human monocytes: Regulation of superoxide anion production and low density lipoprotein oxidation. J. Biol. Chem., 272:2404-2411, 1997.

Mahadevappa, V.G., and Silicial F. Mobilization of arachadonice acid in thrombin-stimulated human platelets. Biochem. Cell. Biol., 68:520-527, 1990.

Mauser, O.J., Pitman, A., Fernandez, X., Zurcher, J., Watnick, A., Egan, R.W., Kreutner, W., Adams III, G.K. The effect of anti-IL-5 on antigen-induced airway hyperreactivity and pulmonary eosinophilia in guinea pigs. Am. Rev. Respir. Dis., 145, A859, 1992.

Moss, J., Tsai, S.C., Bruni, P., Adamik, R., Kanaho, Y., Hewlett, E.L., Vaughan, M. Pertussis toxin-catalyzed ADP-ribosylation of adenylate cyclase. Effects of guanyl nucleotides and rhodopsin. Developments in Biological Standardization, 61, 43-9, 1985.

Motojima, S., Frigas, E., Loegering, D.A., Gleich, G.J. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium *in vitro*. Am. Rev. of Respiratory Disease, 139, 801-5, 1989.

Nagase, T., Ishii, S., Katayama, H., Fukuchi, Y., Ouchi, Y., and Shimizu, T. Airway responsiveness in transgenic mice overexpressing platelet-activating factor receptor. Am. J. Respir. Crit. Care Med., 156:1621-1627, 1997.

Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. Platelet-activating Factor. J. Biol. Chem., 265:17381-17384, 1990.

Rothenberg, M.E., Owen, Jr., W.F., Silberstein, D.S., Woods, J., Soberman, R.J. Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. J. of Clinical Investigation, 81, 1986-92, 1988.

Schleimer, R.P. Effects of glucocorticosteroids on inflammatory cells relevant to their therapeutic applications in asthma. Am. Rev. of Respiratory Disease, 141(2 Pt 2), S59-69, 1990.

Sedgwick, J.B., Calhoun, W.J., Gleich, G.J., Kita, H., Abrams, J.S., Schwartz, L.B., Volovitz, B., Ben-Yaakov, M., Busse, W.W. Immediate and late airway response of allergic rhinitis patients to segmental antigen challenge. Characterization of eosinophil and mast cell mediators. Am. Rev. of Respiratory Disease, 144, 1274-81, 1991.

Sedgwick, J.B., Vrtis, R.F., Gourley, M.F., and Busse, W.W. Stimulus-dependent differences in superoxide anion generation by normal human eosinophils and neutrophils. J. Allergy Clin. Immunol., 81:876-883, 1988.

Sehmi, R., Wardlaw, A.J., Cromwell, O., Kurihara, K., Waltmann, P., Kay, A.B. Interleukin-5 selectively enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects. Blood, 79, 2952-9 (1992).

Silberstein, D.S., Eosinophil function in health and disease. Critical Reviews on Oncology-Hematology, 19, 47-77, 1995.

Silberstein, D.S., Owen, W.F., Gasson, J.C., DiPersio, J.F., Golde, D.W., Bina, J.C., Soberman, R., Austen, K.F., David, J.R. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant)

granulocyte-macrophage colony-stimulating factor. Journal of Immunology, 137, 3290-4, 1986.

Spry, In: Eosinophils, Oxford University Press, pp. 205-212 (1988).

Stewart, A.G., Dubbin, P.N., Harris, T., and Dusting, G.H. Platelet-activating factor may act as a second messenger in the release of icosanoids and superoxide anions from leukocytes and endothelial cells. Proc. Natl. Acad. Sci. USA, 87:3215-3219, 1990.

Takehara, S., Mikashim, H., Muramot, Y., Terasawa, M., Setoguichi, M., and Tahara, T. Pharmacological actions of Y-24180, a new specific antagonist of platelet activating factor (PAF): II. Interactions with PAF and benzodiazepine receptors. Prostaglandins, 40:571-583, 1990..

Terasawa, M., Aratani, H., Setoguchi, M., and Tahara, T. Pharmacological actions of Y-24180: I. A potent and specific antagonist of platelet-activating factor. Prostaglandins, 40:553-569, 1990.

Terashita, Z.C., Imura, Y., Takatani, M., Tsushima, S., and Nishikawa, K. CV-6209, a highly potent antagonist of platelet activating factor *in vitro* and *in vivo*. Journal of Pharmacology and Experimental Therapeutics, 242:242-263, 1987.

Tokumura, A., Homma, H., and Hanahan, D.J. Structural analogs of alkylacetylglycerophosphocholine inhibitory behavior on platelet activation. J. Biol. Chem., 260:12710-12714, 1985.

Trigianni, M. Characterization of platelet-activating factor synthesized by normal and granulocyte-macrophage colony-stimulating factor-primed human eosinophils. Immunology, 77:500-504, 1992.

Uhing, R.J., Perpic, V., Hollenbach, P.W., and Adams, D.O. Involvement of protein kinase C in platelet-activating factor-stimulated diacylglycerol accumulation in murine peritoneal macrophages. J. Biol. Chem., 264:9224-9230, 1989.

Valerius, T., Repp, R., Kalden, J.R., Platzer, E. Effects of IFN on human eosinophils in comparison with other cytokines. A novel class of eosinophil activators with delayed onset of action. Journal of Immunology, 145, 2950-8, 1990.

van der Brugeen, T., Kok, P.T.M., Raaijmakers, J.A.M., Lammers, J.W.J., and Koenderman, L. Cooperation between Fc (receptor II and complement receptor type 3 during activation of platelet-activating factor release by cytokine-primed human eosinophils. J. Immunol., 153:2729-2735, 1994.

Vigo, C., Lewis, G.P., Piper, P.J. Biochemical Pharmacology, 29, 623-7, 1980.

Wardlaw, A.J., Moqbel, R., Cromwell, O., and Kay, A.B. Platelet-activating factor. A potent chemotactic and chemokinetic factor for human eosinophils. J. Clin. Invest., 78:1701-1706, 1986.

White, S.R., Strek, M.E., Kulp, G.V.P., Spaethe, S.M., Burch, R.A., Neeley, S.P., and Leff, A.R. Regulation of human eosinophil degranulation and activation by endogenous phospholipase A_2. J. Clin. Invest., 91:2118-2125, 1993.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. A therapeutic method to inhibit IL-5 induced eosinophil activation or degranulation, comprising: administering to a mammal in need of said therapy an effective amount of an inhibitor of platelet activating factor.

2. A method to treat an eosinophil-associated pathology, comprising: administering to a mammal in need of said therapy a dosage form comprising an effective amount of an inhibitor of platelet activating effective to counteract at least one symptom of the pathology, wherein the dosage form is bound to a binding peptide or protein that specifically binds to eosinophils.

3. A method for treating bronchial asthma, comprising: administering to the respiratory tract of a mammal afflicted with bronchial asthma an amount of factor an inhibitor of platelet activating factor effective to counteract the symptoms of bronchial asthma by inhibiting the degranulation of IL-5 activated eosinophils.

4. A method for treating bronchial asthma, comprising: administering to the respiratory tract of a mammal afflicted with bronchial asthma an amount of factor an inhibitor of platelet activating factor effective to counteract the symptoms of bronchial asthma by inhibiting IL-5 induced activation of eosinophils.

5. A therapeutic method to inhibit immunoglobulin induced eosinophil activation or degranulation, comprising: administering to a mammal in need of said therapy an effective amount of an inhibitor of platelet activating factor.

6. A therapeutic method comprising: administering to a mammal having an eosinophil-associated disease an amount of an inhibitor of platelet activating factor effective to inhibit or reduce eosinophil activation or degranulation.

7. The method of any one of claims 1 to 6 wherein the inhibitor is a platelet activating factor receptor antagonist.

8. The method of any one of claims 1 to 7 wherein antagonist is administered by spraying or nebulization.

9. The method of claim 8 wherein the administration is to the nose, respiratory tract or mouth.

10. The method of claim 2 wherein the dosage form is locally administered.

11. The method of any one of claims 1 to 7 wherein the inhibitor is systemically administered.

12. The method of any one of claims 1 to 11 wherein the inhibitor is administered in combination with a pharmaceutically acceptable carrier.

13. The method of claim 12 wherein the inhibitor and carrier are administered in a unit dosage form.

14. The method of claim 2 wherein the dosage form is adapted for oral administration.

15. The method of claim 2 wherein the dosage form is adapted for administration by inhalation.

16. The method of claim 6 wherein the activation or degranulation is induced by IL-5.

17. The method of claim 5 wherein sIgA induced degranulation is inhibited.

18. The method of claim 16 wherein the degranulation is induced by the secretory component of sIgA.

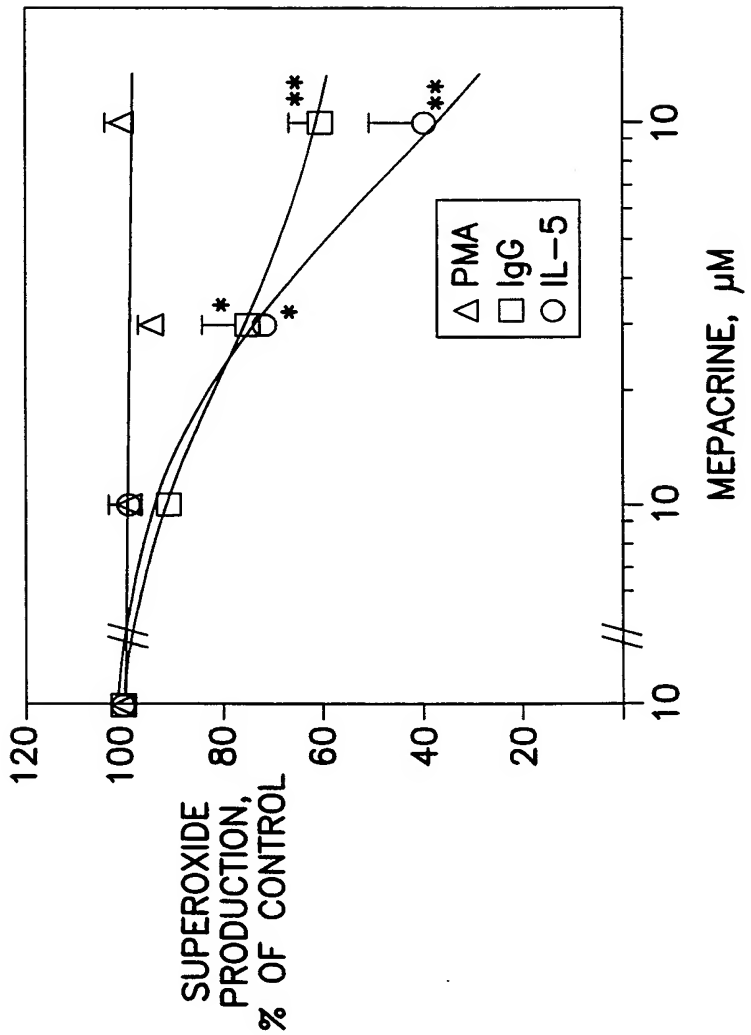

SUPEROXIDE PRODUCTION, % OF CONTROL
120
100
80
60
40
20
10
10
10
MEPACRINE, μM
△ PMA
□ IgG
○ IL-5
*
**
**

FIG. 1

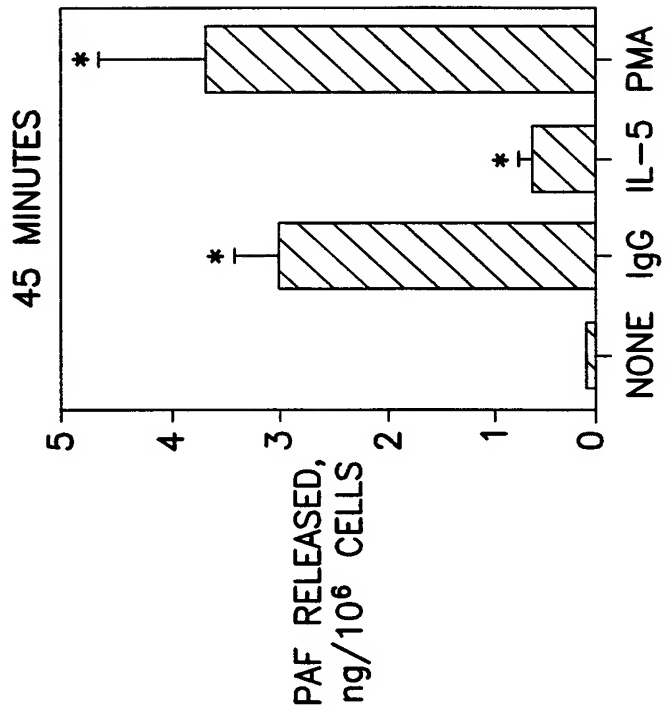

45 MINUTES
PAF RELEASED, ng/10⁶ CELLS
0
1
2
3
4
5
*
*
*
NONE
IgG
IL-5
PMA
AGONIST

FIG. 2B

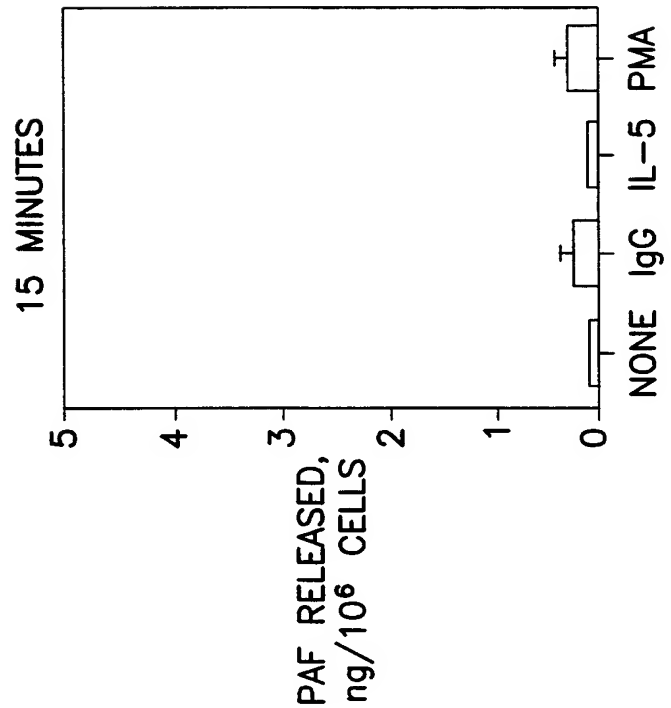

15 MINUTES
PAF RELEASED, ng/10⁶ CELLS
0
1
2
3
4
5
NONE
IgG
IL-5
PMA
AGONIST

FIG. 2A

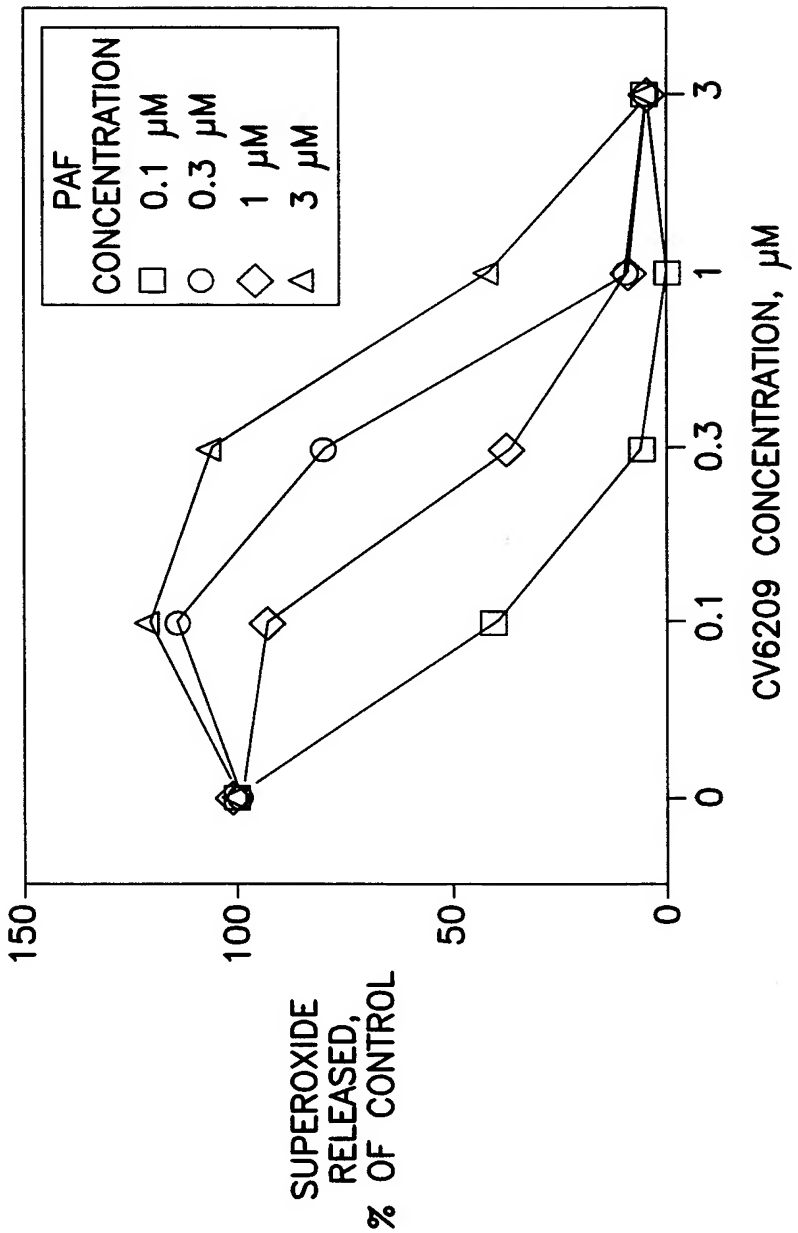

PAF CONCENTRATION
0.1 μM
0.3 μM
1 μM
3 μM
SUPEROXIDE RELEASED, % OF CONTROL
150
100
50
0
0
0.1
0.3
1
3
CV6209 CONCENTRATION, μM

FIG. 3

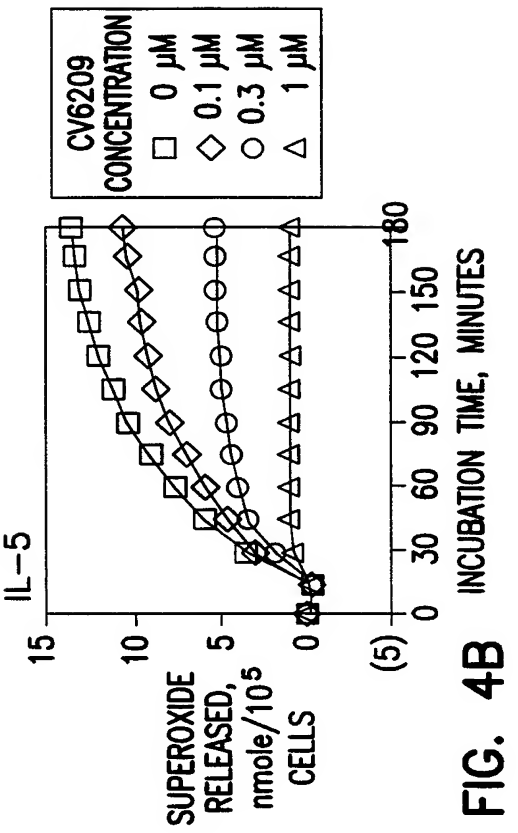


FIG. 4B

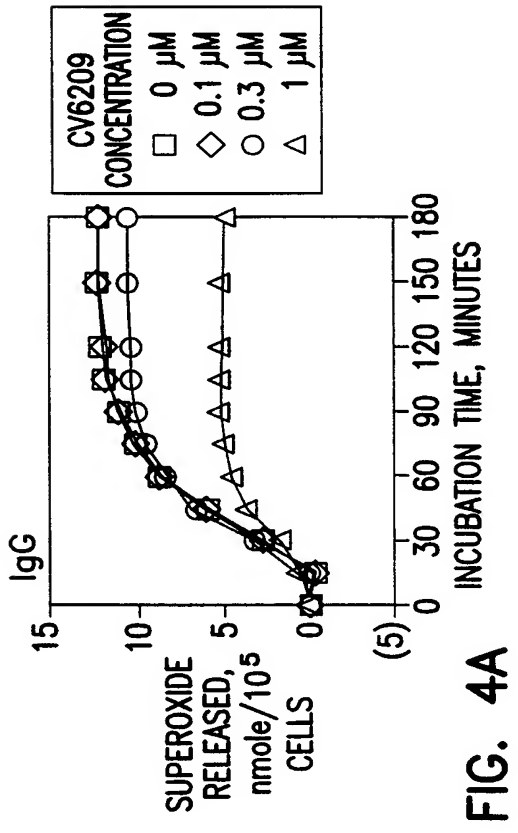


FIG. 4A

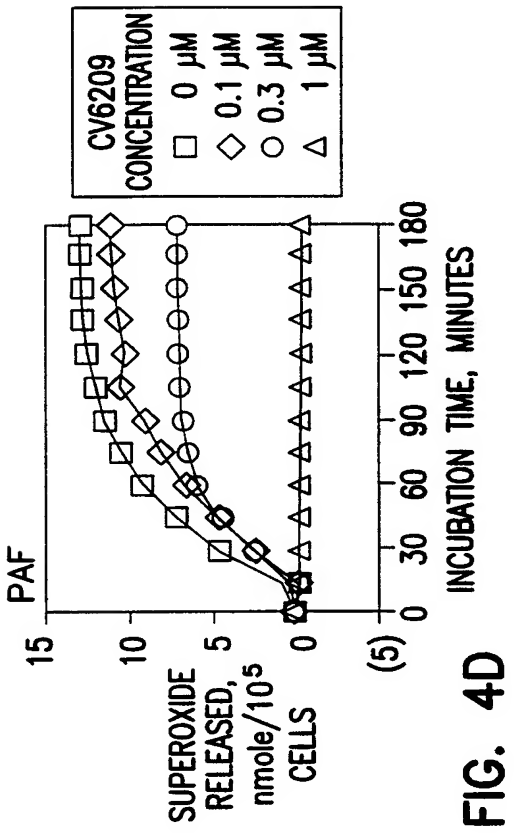


FIG. 4D

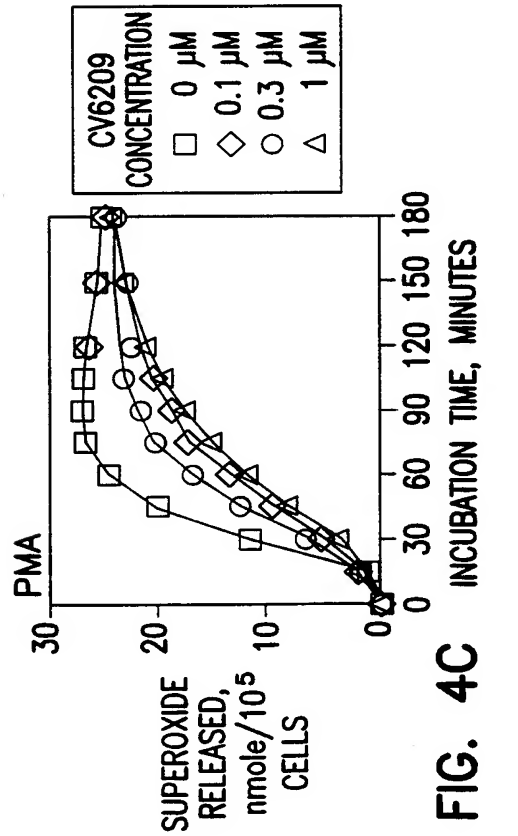


FIG. 4C

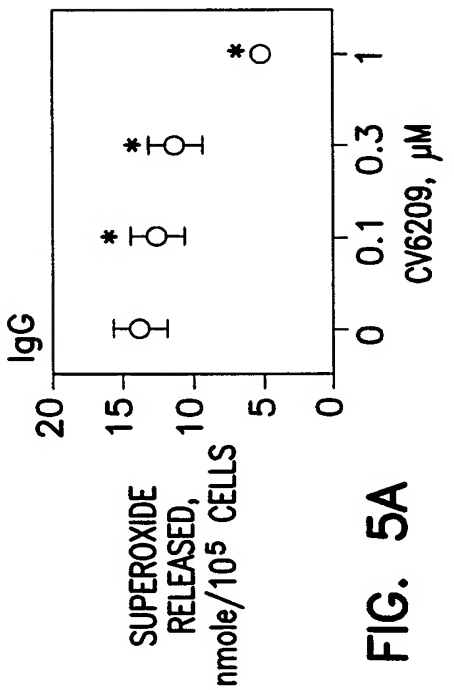


FIG. 5A

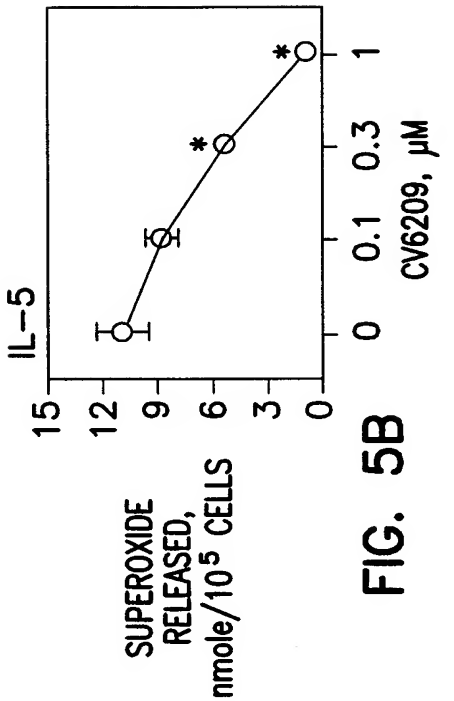


FIG. 5B

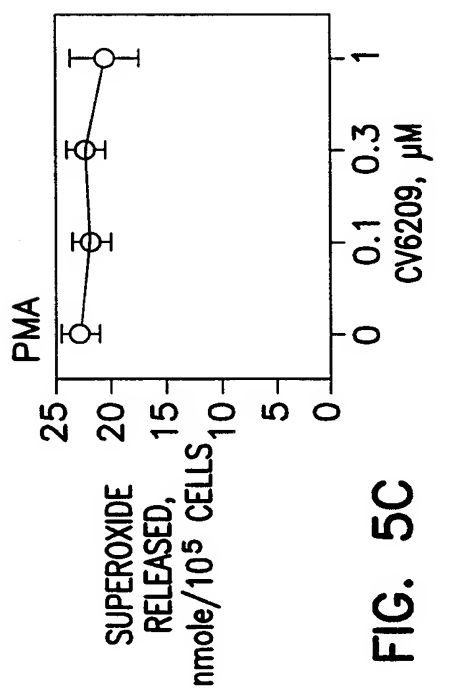


FIG. 5C

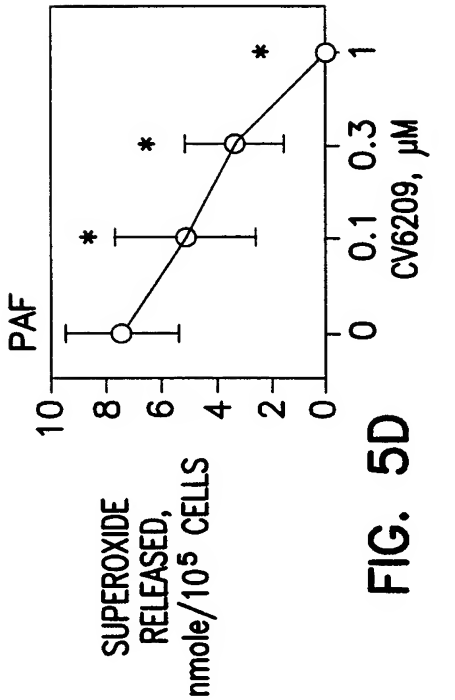


FIG. 5D

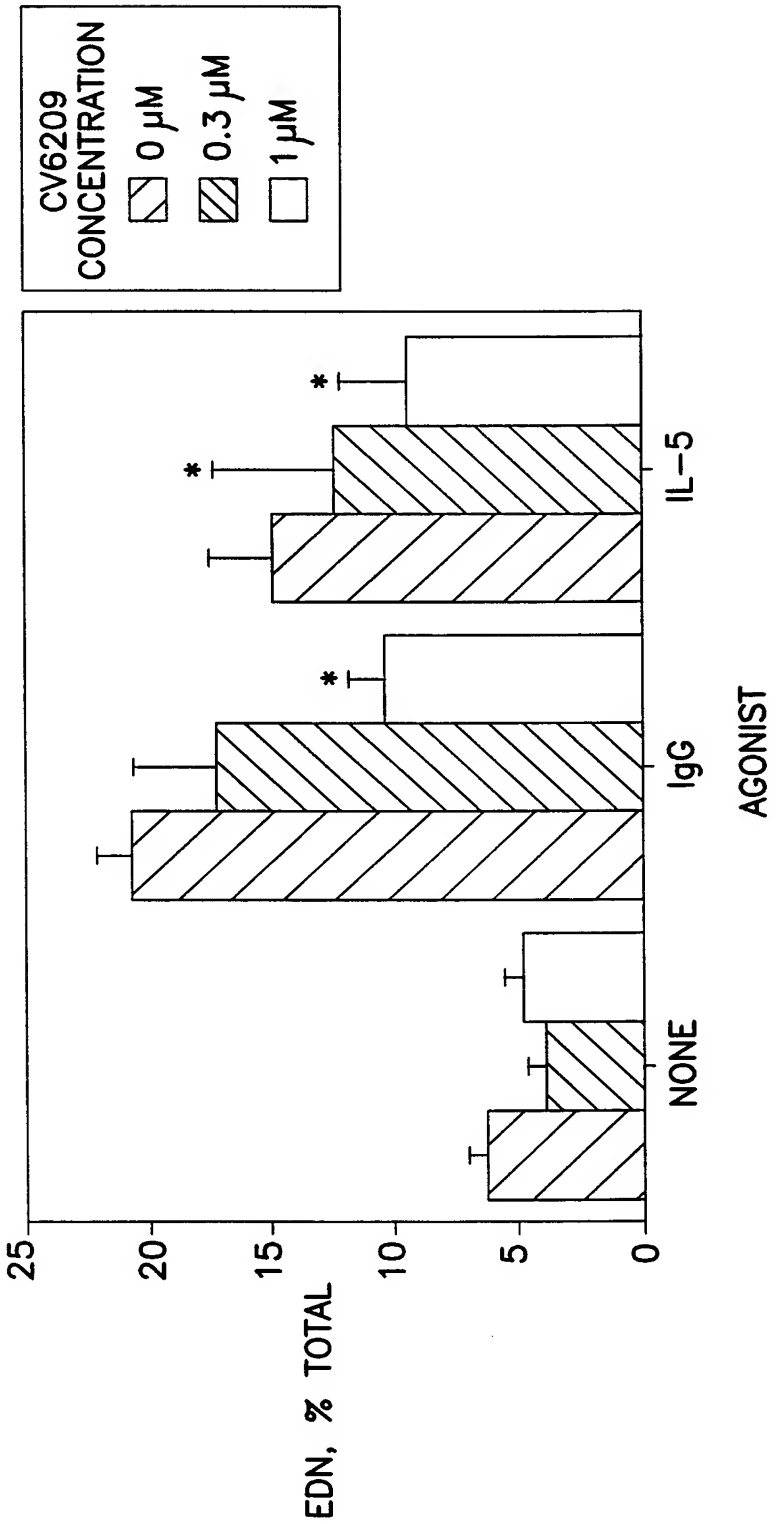

CV6209
CONCENTRATION
0 μM
0.3 μM
1 μM
EDN, % TOTAL
25
20
15
10
5
0
NONE
IgG
IL-5
AGONIST
*
*
*

FIG. 6

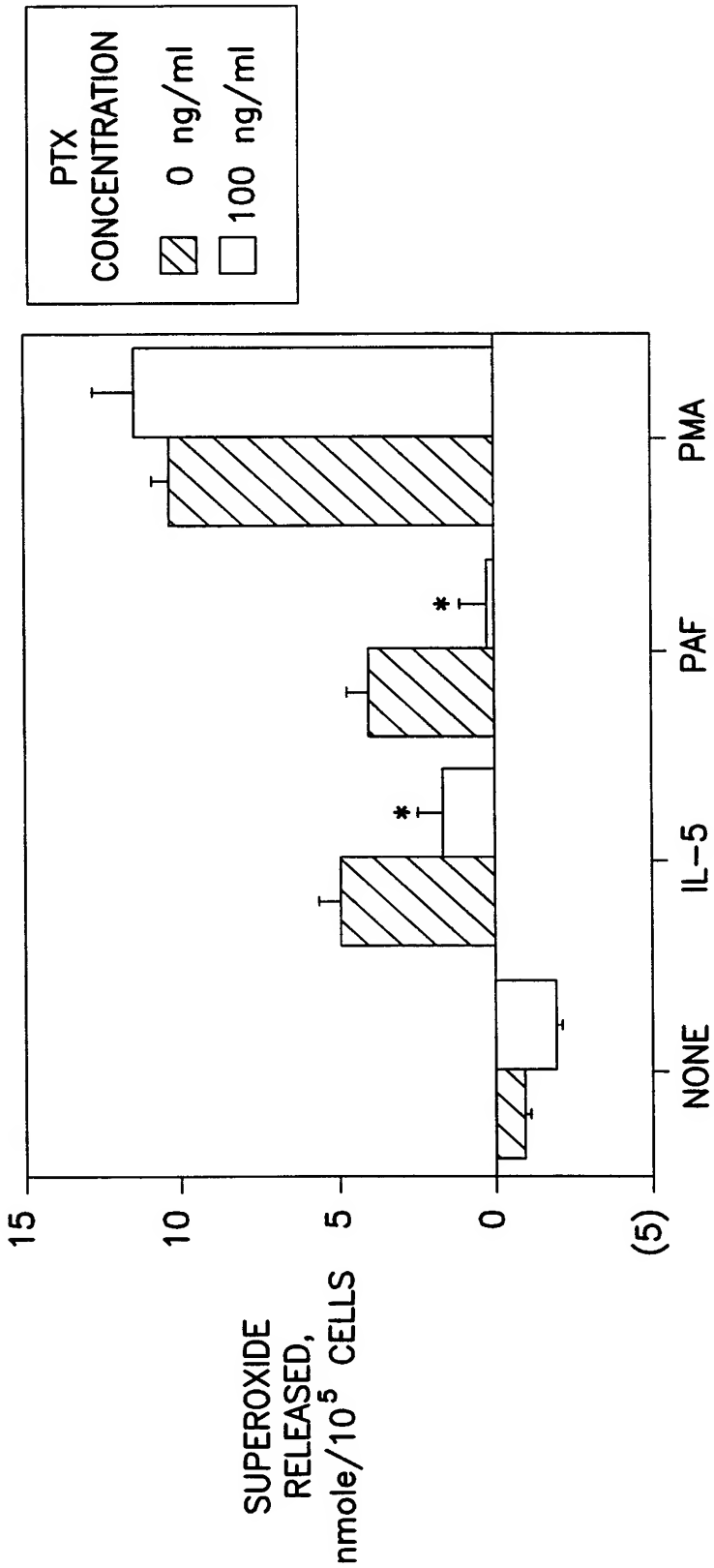

PTX CONCENTRATION
0 ng/ml
100 ng/ml
SUPEROXIDE RELEASED, nmole/10^5 CELLS
15
10
5
0
(5)
NONE
IL-5
PAF
PMA
AGONIST
*
*

FIG. 7

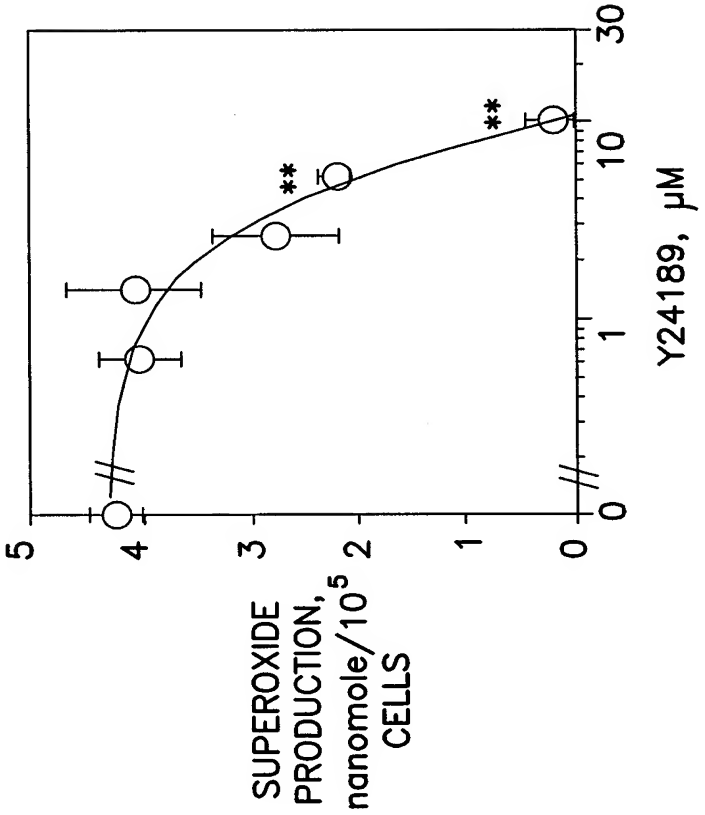

SUPEROXIDE PRODUCTION, nanomole/10^5 CELLS
0
1
2
3
4
5
0
1
10
30
Y24189, μM
**
**
**

FIG. 8B

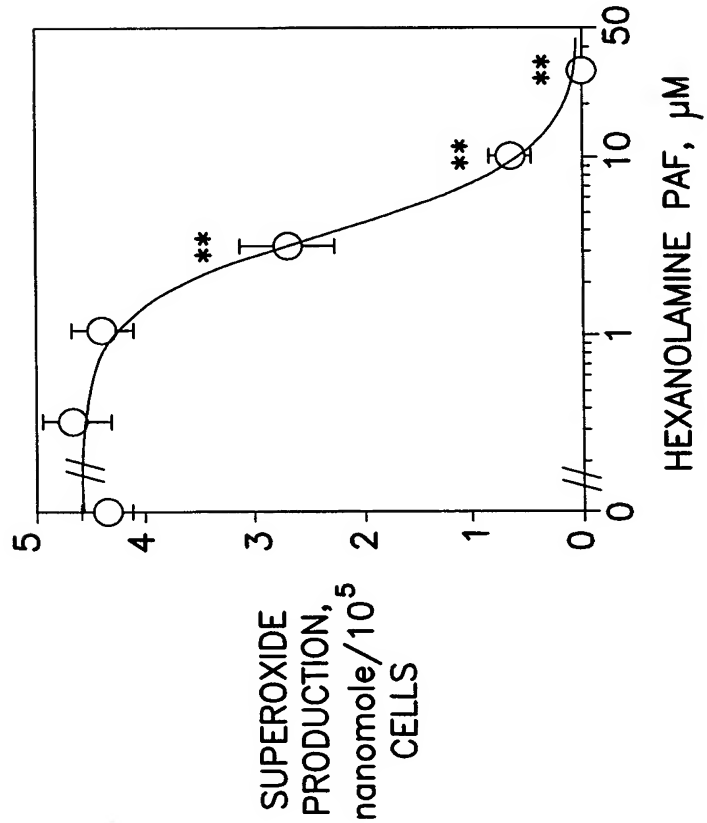

SUPEROXIDE PRODUCTION, nanomole/10^5 CELLS
0
1
2
3
4
5
0
1
10
50
HEXANOLAMINE PAF, μM
**
**
**

FIG. 8A

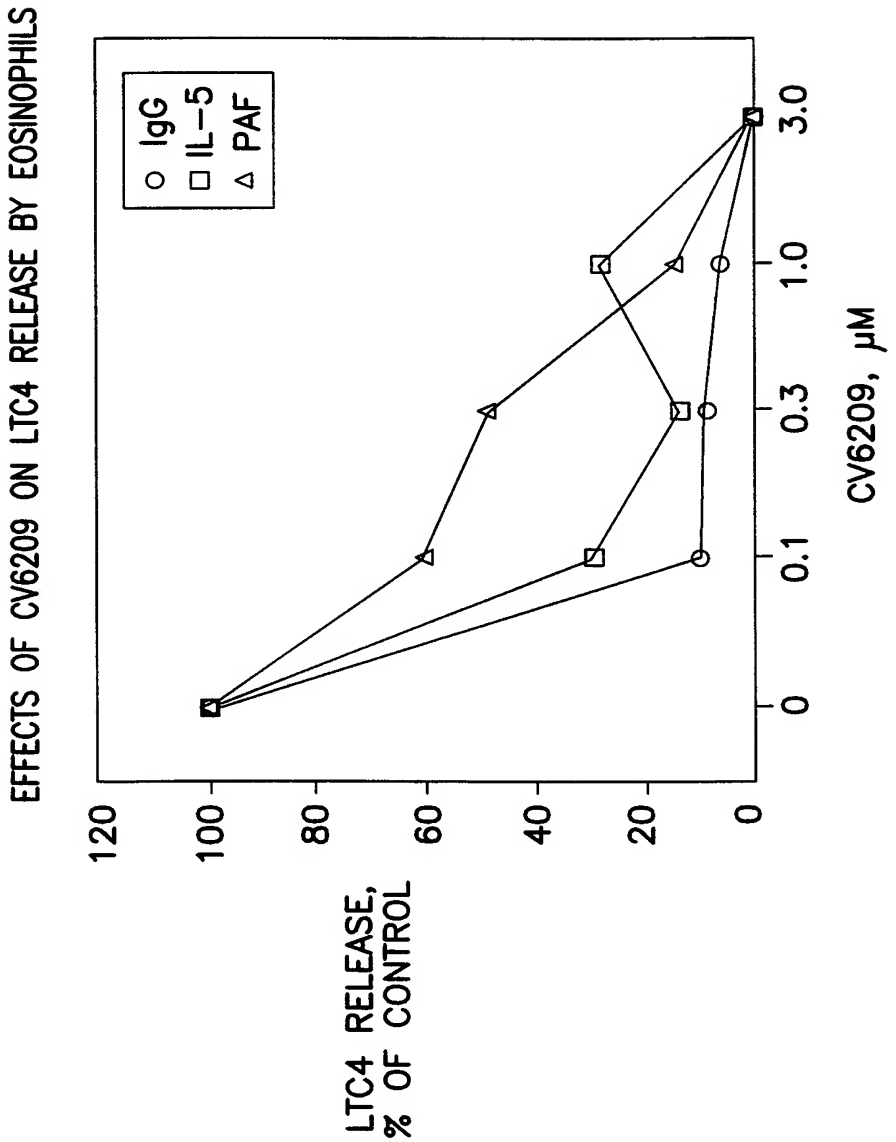

EFFECTS OF CV6209 ON LTC4 RELEASE BY EOSINOPHILS
IgG
IL-5
PAF
LTC4 RELEASE, % OF CONTROL
120
100
80
60
40
20
0
0
0.1
0.3
1.0
3.0
CV6209, μM

FIG. 9

INTERNATIONAL SEARCH REPORT

Int(ional Application No
PCT/US 99/06859

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/445

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 369 810 A (TAKEDA CHEMICAL INDUSTRIES LTD) 23 May 1990 (1990-05-23) page 2, line 8-28 page 7, line 35-50 page 26, line 25-44 page 29, line 52 - page 34, line 20; claim 2	1-6, 16-18
X	EP 0 540 766 A (KORTH RUTH) 12 May 1993 (1993-05-12) the whole document	1-6, 16-18
X	EP 0 549 364 A (SANKYO CO) 30 June 1993 (1993-06-30) the whole document	1-18

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 August 1999	18/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Engl, B

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INTERNATIONAL SEARCH REPORT

Int[illegible]ional Application No
PCT/US 99/06859

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 18131 A (SUNKYONG IND LTD) 6 July 1995 (1995-07-06) page 1, line 14 - page 4, line 19 page 18, line 23 - page 19, line 7 page 60, line 24 - page 63, line 7 page 64, line 10 - page 74, line 7 ---	1-18
X	EP 0 686 635 A (SANKYO CO) 13 December 1995 (1995-12-13) page 2, line 1-14 page 5, line 33-42 page 58, line 8 - page 61, line 10 ---	1-6, 16-18
X	WO 96 00212 A (CYTOMED INC) 4 January 1996 (1996-01-04) page 1, line 4-6 page 2, line 30 - page 5, line 11 page 6, line 1-8 page 9, line 33 - page 10, line 15 page 78, line 22 - page 87, line 30 page 98, line 7 - page 102, line 35 ---	1-18
X	WO 96 08496 A (TAKEDA CHEMICAL INDUSTRIES LTD) 21 March 1996 (1996-03-21) page 1, line 5-9 page 38, line 12 - page 41, line 29 page 43, line 2-6 ---	1-6, 16-18
X	US 5 703 062 A (NAIR HARIDASAN K) 30 December 1997 (1997-12-30) column 1, line 58 - column 2, line 19 column 6, line 18 - column 8, line 5 column 8, line 6 - column 10, line 31 ---	1-18
X	DATABASE WPI Section Ch, Week 9743 Derwent Publications Ltd., London, GB; Class B02, AN 97-466223 XP002111393 & JP 09 216886 A (TAKEDA CHEM IND LTD), 19 August 1997 (1997-08-19) abstract ---	1-6, 16-18
X	DATABASE WPI Section Ch, Week 9750 Derwent Publications Ltd., London, GB; Class B02, AN 97-545513 XP002111394 & JP 09 263586 A (TAKEDA CHEM IND LTD), 7 October 1997 (1997-10-07) abstract ---	1-6, 16-18
Y	WO 94 17790 A (MAYO FOUNDATION) 18 August 1994 (1994-08-18) page 1, line 10 - page 5, line 23 ---	1-18
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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/06859

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 26727 A (HOECHST AG ;GEBERT ULRICH (DE); ANAGNOSTOPULOS HIRISTO (DE); KROEG) 12 October 1995 (1995-10-12) page 2, line 26 - page 4, line 7 page 34, line 1 - page 40, line 14 ---	1-18
Y	WO 96 32481 A (INCYTE PHARMA INC) 17 October 1996 (1996-10-17) page 3, line 5 - page 4, line 29 -----	1-18

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ional Application No
PCT/US 99/06859

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0369810 A	23-05-1990	CA 2003210 A	18-05-1990
		JP 2231433 A	13-09-1990
EP 0540766 A	12-05-1993	US 5696114 A	09-12-1997
		US 5895785 A	20-04-1999
EP 0549364 A	30-06-1993	JP 5255334 A	05-10-1993
		AT 144515 T	15-11-1996
		AU 649366 B	19-05-1994
		AU 3043292 A	01-07-1993
		CA 2086213 A	28-06-1993
		CN 1074682 A,B	28-07-1993
		CN 1130630 A	11-09-1996
		CZ 9203898 A	19-01-1994
		DE 69214791 D	28-11-1996
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		DK 549364 T	24-03-1997
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		FI 925850 A	28-06-1993
		GR 3021691 T	28-02-1997
		HU 67733 A	28-04-1995
		NZ 245576 A	26-07-1995
		US 5470851 A	28-11-1995
		JP 5239056 A	17-09-1993
		MX 9207596 A	01-06-1994
		ZA 9210022 A	02-07-1993
WO 9518131 A	06-07-1995	AU 1426095 A	17-07-1995
		BR 9408463 A	26-08-1997
		CA 2177736 A	06-07-1995
		CN 1139435 A	01-01-1997
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		JP 9506107 T	17-06-1997
		KR 136986 B	25-04-1998
		PL 315174 A	14-10-1996
		US 5541183 A	30-07-1996
EP 0686635 A	13-12-1995	JP 5255334 A	05-10-1993
		AT 144515 T	15-11-1996
		AU 649366 B	19-05-1994
		AU 3043292 A	01-07-1993
		CA 2086213 A	28-06-1993
		CN 1074682 A,B	28-07-1993
		CN 1130630 A	11-09-1996
		CZ 9203898 A	19-01-1994
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		ES 2096050 T	01-03-1997
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		GR 3021691 T	28-02-1997
		HU 67733 A	28-04-1995
		JP 5239056 A	17-09-1993
		MX 9207596 A	01-06-1994
		NZ 245576 A	26-07-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]ional Application No
PCT/US 99/06859

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0686635 A		US 5470851 A	28-11-1995
		ZA 9210022 A	02-07-1993
WO 9600212 A	04-01-1996	US 5792776 A	11-08-1998
		US 5750565 A	12-05-1998
		US 5703093 A	30-12-1997
		AU 703756 B	01-04-1999
		AU 2914295 A	19-01-1996
		BR 9508196 A	02-09-1997
		CA 2194064 A	04-01-1996
		CZ 9603720 A	15-04-1998
		EP 0770064 A	02-05-1997
		FI 965123 A	26-02-1997
		HU 77773 A	28-08-1998
		JP 10506096 T	16-06-1998
		NO 965569 A	17-02-1997
		PL 317873 A	28-04-1997
		US 5780503 A	14-07-1998
		US 5681966 A	28-10-1997
WO 9608496 A	21-03-1996	US 5482939 A	09-01-1996
		AU 695377 B	13-08-1998
		AU 3399995 A	29-03-1996
		CA 2198019 A	21-03-1996
		CN 1158130 A	27-08-1997
		EP 0781283 A	02-07-1997
		FI 971081 A	06-05-1997
		HU 77130 A	02-03-1998
		JP 9328485 A	22-12-1997
		JP 8198878 A	06-08-1996
		NO 971117 A	11-03-1997
		NZ 292271 A	27-04-1998
US 5703062 A	30-12-1997	NONE	
JP 9216886 A	19-08-1997	NONE	
JP 9263586 A	07-10-1997	NONE	
WO 9417790 A	18-08-1994	US 5510339 A	23-04-1996
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		AU 6128294 A	29-08-1994
		CA 2155200 A	18-08-1994
		EP 0682514 A	22-11-1995
		JP 8505157 T	04-06-1996
		NZ 261990 A	22-08-1997
		US 5631267 A	20-05-1997
WO 9526727 A	12-10-1995	DE 4411660 A	12-10-1995
		AU 695488 B	13-08-1998
		AU 1951095 A	23-10-1995
		CA 2187081 A	12-10-1995
		CN 1145029 A	12-03-1997
		EP 0758239 A	19-02-1997
		HU 74732 A	28-02-1997
		JP 9510983 T	04-11-1997
WO 9632481 A	17-10-1996	US 5874211 A	23-02-1999

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 99/06859

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9632481 A		AU 5543496 A	30-10-1996
		CA 2217025 A	17-10-1996
		EP 0820512 A	28-01-1998
		JP 11503610 T	30-03-1999